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The Absorption, Metabolism and Excretion of the Sulphonated Azo Dye, Acid Yellow by Rats

By

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(Received February 25 1965)

The widespread use of coal-tar colours in the food industry makes it essential that the biochemical, pharmacological and toxicological properties of these substances should be known. WILLIAMS (1959) stated that the information ideally needed about any compound used as a food additive includes knowledge of its toxicity absorption metabolism, elimination and accumulation.

The commonly used red, yellow and orange food colours are sulphonated azo dyes until recently little information has been available in the literature about their absorption metabolism and excretion. A few recent publications help to remedy this shortcoming, and DANIEL (1962) reported on the metabolism and excretion of 11 azo dyes sulphonated on one or both sides of the azo linkage He found that only a few per cent of the original colour was excreted in the urine of rabbits after oral dosage, and various amines and their derivatives resulting from the reductive fission of the azo linkage were present in the 48 hour urine samples corresponding to 60–80 / of the dose.

Evidence for this extensive splitting of azo dyes has also been presented by RADOMSKI & MELLINGER (1962), who made a detailed study of the absorption, metabolism and excretion of Amaranth, Ponceau SX and Sunset Yellow by rats. They found all the component amines in the urine and obtained results indicating that these originated from their absorption from the gastrointestinal tract after bacterial reduction of the dyes. Recently JONES *et al.* (1964) have reported on the metabolism of Tartrazine in the rat, rabbit and man, and they found little or no urinary excretion of the dye after oral administration Large amounts of sulphanilic acid were found in the urine, presumably formed from reduction of the dye by the gastrointestinal flora.

Acid Yellow (Colour Index 1956 No 13015, Fast Yellow Egg Yellow) is permitted as a food colour in several countries including Belgium, *Italy The Netherlands, Norway* and Western Germany Its structure is shown in Fig. 1

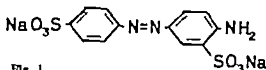


Fig. 1

Except for the finding by Ryan & Wright (1961) that small amounts of Acid Yellow were excreted in the bile of rats after its intravenous injection, no reports have appeared in the literature about its biological properties We report here on the absorption, metabolism and excretion of Acid Yellow after oral dosage to rats.

Methods

Animals The rats used in these experiments were adult albino males weighing from 230 to 350 g. They were allowed free access to food and water before and during all experiments. Light ether anaesthesia was used when the compounds were given by oral or intraperitoneal administration, and nebutal sodium (pentobarbital), 35 mg/kg intraperitoneally was used as anaesthetic in the biliary studies.

Chemicals. Acid Yellow was obtained from Farbenfabriken Bayer AG and was an extra pure product intended for use as a food colour This normally gave a single spot on chromatograms however when large amounts were applied, a faint yellow spot of less polar nature was seen. Spectrophotometric estimation showed that this substance represented 0.07 % of the total amount of colour present. The amount of soluble inorganic material present was determined by estimating the amount of sulphanilic acid formed from known amounts of the dye as a result of reductive fission of the azo linkage. The purity was found to be 66 % and the theoretical recoveries of the metabolites of Acid Yellow are based on this value Sulphanilic acid was obtained from E. Merck AG and p-phenylenediamine sulphonic acid dihydrate from The British Drug Houses Ltd. Both of the monoacetyl derivatives of p-phenylenediamine sulphonic acid were prepared by the method of Jarkovsky & Allan (1959). The N-acetyl derivative of sulphanilic acid was prepared by warming an ethanolic solution of sulphanilic acid with acetic anhydride, cooling and recrystallizing the product from ethanol-ether

Chromatographic methods Urine, bile and faeces extract samples, together with appropriate standards, were examined by ring chromatography on Whatman No 1 paper or by thin layer chromatography on 0.3–0.5 mm thick plates of cellulose (MN 300, Macherey Nagel & Co) Rf values of Acid Yellow and related derivatives are shown in table 1 The amino compounds were visualized by spraying with Ehrlich reagent (50 mg p-dimethylaminobenzaldehyde dissolved in 10 ml ethanol containing 0.1 ml concentrated HCl) The colours obtained are described in table 2.

Determination of Acid Yellow and metabolites in urine The urine and faeces were collected separately in containers placed in solid carbon dioxide, the separator and metabolism cage being those described by Naa (1963). Acid Yellow was determined colorimetrically by adding an equal volume of 4N–HCl to a suitably diluted sample and reading the optical

Table I

Rf values of Acid Yellow and related derivatives

Compound	Thin layer chromatography (cellulose)				Ring chromatography (Whatman no 1 paper)	
	Solvent system				Solvent system	
	A	B	C	D	A	B
Acid Yellow	0.28	0.21	0.17	0.03	0.31	0.14
Sulphanilic acid	0.27	0.37	0.26	0.11	0.36	0.31
N-Acetyl sulphanilic acid	0.36	0.53	0.38	0.23	–	–
p-Phenylenediamine sulphonic acid	0.16	0.24	0.16	0.10	0.24	0.16
5-Acetylamino-2-aminobenzene sulphonic acid	0.34	0.43	0.32	0.17	0.48	0.35
2-Acetylamino-5-aminobenzene sulphonic acid	0.41	0.49	0.36	0.24	–	–

Solvent system

A pyridine – ethyl acetate – 0.2 M acetic acid (1 2 2, upper phase)
B n-propanol dioxan – 0.2 M acetic acid (3 1 1)
C n-butanol dioxan – 5 N NH_3 (3:1 1)
D n-butanol glacial acetic acid – water (4 1:1)

density at the absorption maximum of 498 mμ on a Beckman model DB double-beam spectrophotometer. The reference solution was obtained by reducing the dye in 10 ml of the sample with 10 mg of $SnCl_2$.

Sulphanilic acid was determined before and after acid hydrolysis by a slight modification of the method of BRATTON & MARSHALL (1939). To 1 ml sample were added 1 ml 2 N–HCl (total sulphanilic acid was obtained by heating the acidified sample in a boiling water bath for 30 min) and then 1 ml 0.25 N $NaNO_2$. After 10 min., 1 ml 0.5% ammonium sulphamate was added and after a further 2 min., 1 ml of 0.1% N-(1-naphthyl) ethylenediamine. The sample was diluted to 10 ml and the optical density measured at 545 mμ. Normal urine collected during the two days before dosing was assayed for each rat and served as a blank. These values were subtracted from the results obtained for the 24, 48 and 72 hour samples.

p-Phenylenediamine sulphonic acid was determined fluorimetrically in an Aminco-Bowman Spectrophotofluorometer model 4–8202 SPF. The relationship between relative fluorescence intensity and concentration was linear over the range of 0.02–10 μg/ml when using 0.1 M phosphate buffer (pH 8), instrument settings of 321 mμ (excitation maximum) and 428 mμ (fluorescence maximum) and slit arrangement no. 3. Samples of 1 ml, before and after heating with an equal volume of 2 N–HCl for 30 min. on a boiling water bath, were suitably diluted with phosphate buffer and the fluorescence intensity was measured. As normal urine was fluorescent under these conditions, the urine collected during the two days before and on the third after dosing was used to determine the blank fluorescence for each rat, and this value was subtracted from the results obtained for the 24 and 48 hour samples.

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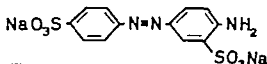


Fig. 1

Except for the finding by RYAN & WRIGHT (1961) that small amounts of Acid Yellow were excreted in the bile of rats after its intravenous injection no reports have appeared in the literature about its biological properties. We report here on the absorption, metabolism and excretion of Acid Yellow after oral dosage to rats.

Methods

Animals The rats used in these experiments were adult albino males weighing from 250 to 350 g. They were allowed free access to food and water before and during all experiments. *Light ether anaesthesia was used when the compounds were given by oral or intraperitoneal* administration, and mebumal sodium (pentobarbital), 35 mg/kg intraperitoneally was used as anaesthetic in the biliary studies.

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Solvent system

A. pyridine – ethyl acetat – 0.2 M acetic acid (1 2 2, upper phase)
B n-propanol – dioxan – 0.2 M acetic acid (3 1:1)
C: n-butanol – dioxan – 5% NH_3 (3 1 1)
D n-butanol – glacial acetic acid – water (4 1 1)

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Table 2

Characteristics f Acid Yellow and related derivatives

Compound	V sible colou	Colour with Ehrlich s reagent	Ultraviolet absorption maxima pH 8 (minor peak in parentheses) mμ	Fluorescence[1] pH 8 Excitation maxima mμ	Fluorescence maxima mμ
Acid Yellow	yellow	red-orange	384	–	no fluorescence
Sulphanilic cid	none	yellow	248	.50	343
N Acetyl sulphanilic acid	none	yellow after a few hours	250	–	no fluorescence
p-Phenylenediamine sulphonic acid	none	red	247(322)	321	428
5-Acetylamino-2-amino-benzene sulphonic acid	none	ora ge	257(304)	310	388
2 Acetylamino-5-amino-benzene sulphonic acid	none	yellow-range	251(298)	302	396

[1]) nstrument alues, see Methods.

De ermlnatio of Acid Yello ad metabolites in faeces. The combined 48 hour faeces samples were homogenized with 20 ml of water f r each 5 g f faeces nd centrifuged. A total of five such extractions was made, nd the pooled supernatants were centrifuged at 20,000 g f 10 min. in Servall refrigerated centrifuge. A measured portio f the resulting supernatant was heated with an equal vol me f ethanol for five min. in boiling water bath, cooled and centrifuged t 20,000 g for 30 min. This method, which is similar to that described by RADOMSKI & MELLINGER (1962), resulted in clear supernatant suitable for colorimetric or fluorimetric assay Determinations f Acid Yellow and p-phenylenediamine sulphonic cid in the faeces extract were carried out as described bove. The colorimetric assay f sulphanilic acid was affected by the large amounts f p-phenylenediamine sulphonic acid present, nd a fluorimetric assay (0.1 M phosphat buffer pH 8, excitation 251 mμ, fluorescence 343 mμ and sl t arrangement no 3) was therefore used. The relationship between fluorescence intensity and concentration was linear over the range of 0.1 2 μg/ml. The faeces collected for 48 hours before dosing was similarly assayed to btain blank values for sulphanilic acid and p-phenylenediamine sulphonic acid for each rat.

Determination of Acid Yellow in bile The common bile duct as cannulated ith thin plastic t bing, which was led to the outside laterally through the body wall. The rats were placed in restraining cage (SCHELINE 1965), and the bile and urine were collected in test tubes maintained at −2–4°C

The Acid Yellow present in the bile was determined by the method of RYAN & WRIGHT (1961). T 4 ml bile were added 3 ml 40% $ZnSO_4$ and then 3 ml NaOH. After mixi g, the sample was centrifuged d decanted, and the precipitate was washed twice with 3 ml portions of warm water The volume was adjusted to 15 ml, and Acid Yellow was deter mined as described above. A haziness often developed on acidification, but this could be removed by centrifugation. This method gave recovery of 96% when known amounts of Acid Yellow were added to normal bile samples.

Results

The 24 hour urines of all rats given Acid Yellow orally (100 mg in 1 ml water by stomach tube) contained the unchanged dye (table 3) This was confirmed chromatographically before and after spraying with Ehrlich s reagent. Acid Yellow was not found in the urine voided after 24 hours, and none was found in the faeces.

Besides the observed urinary excretion of 1 1 %, the total gastrointestinal absorption of the dye will include any quantity excreted into the bile as well as th products of the absorbed dye due to splitting by azo reduc tase (FOUTS *et al* 1957) The biliary excretion is shown in table 4 the results indicate that this is a minor excretory route, accounting for only about 5 / of the amount found in the urine. Chromatograms of these bile samples differed from controls only in the presence of a single spot, which corresponded in Rf value and color to Acid Yellow The urinary excretion of Acid Yellow was consistently higher in these rats than in those not operated upon (table 3) It was also noted that the latter group excreted a highly colored urine during the first few hours after dosing, though this

Table 3

Excretion f unchanged Acid Yellow in the urine and faeces of rats after oral dosage with 100 mg of Acid Yellow

Rat no	0–24 hour urine %	24–48 hour urine %	0–48 hou faeces %	Total %
1	1 2	0	0	1.2
2	1)	0	0	1)
3	1 0	0	0	1.0
4	0.6	0	0	0.6
5	1.2	0	0	1.2
6	1.5	0	0	1.5
7	1.2	0	0	1..
Mean	1 1	0	0	1 1
e.m.	±0.1	–		±0.1

1) present but not determined.

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Excretion of unchanged Acid Yellow in the urine and faeces of rats fter oral dosage with 100 mg of Acid Yellow

Rat no.	0–24 hour urine %	24–48 hour urine %	0–48 hour faeces %	Total %
1	1.2	0	0	1.2
2	)	0	0	)
3	1.0	0	0	1.0
4	0.6	0	0	0.6
5	1.2	0	0	1.2
6	1.5	0	0	1.5
7	1.2	0	0	1.2
Mean	1.1	0	0	1.1
s.e.m.	±0.1		–	±0.1

) present but not determined.

Table 4

Excretion of unchanged Acid Yellow in the bile and urine of rats after oral dosage with 100 mg of Acid Yellow

Rat no.	0–24 hour bile %	0–24 hour urine %	Total %
1	0.13	2.1	2.2
2	0.10	2.5	2.6
3	0.12	3.1	3.2
Mean	0.12	2.6	2.7
s.e.m.	±0.009	±0.3	±0.3

became normal in color before 24 hours. The rats in the biliary studies showed a prolonged excretion of the dye, and small amounts were seen as late as 24 hours after dosing.

The possibility of reduction of the absorbed Acid Yellow by the azo reductase system was studied in a group of three rats. These were given an intraperitoneal dose of 2 mg dye in saline, and the urine and faeces were studied for presence of the dye and possible metabolites. The recovery of unchanged dye in the 24 hour urine was 87 %, and none was found in the 48 hour urine or the 0–48 hour faeces. Chromatographic and fluorimetric examination of these samples failed to reveal the presence of any metabolites. Another rat was given 1.5 mg dye by intrasplenic infusion (2 hours) and the 24 hour urine and bile samples were collected. The amount of unchanged dye found in the bile was about 3 % of that found in the 24 hour urine and neither sample contained any metabolites. These results appear to rule out the importance of azo reductase in the metabolism of Acid Yellow and indicate that the urinary excretion value of 1.1 % closely represents the amount of the dye absorbed from the gastrointestinal tract of normal rats.

The chromatograms of urine and faeces after Acid Yellow administration showed with Ehrlich's reagent several prominent spots not seen in samples from untreated animals. The two most prominent of these corresponded in color and Rf values to sulphanilic acid and p-phenylenediamine sulphonic acid. further samples diluted with pH 8 phosphate buffer displayed the excitation and fluorescence characteristics of these compounds (table 2). The results of the quantitative determination of these compounds after dosage with Acid Yellow are given in tables 5 and 6 (the same animals as table 3) which show that the excretion of metabolites was essentially complete within 48 hours. Traces of p-phenylenediamine sulphonic acid were sometimes seen in the faeces collected

Table 5

Excretion of sulphanilic acid in the urine and faeces of rats after oral dosage with 100 mg Acid Yellow

Rat no.	0–24 hour urine		24–48 hour urine		48–72 hour urine		Total urine		0–48 hour faeces		Total urine and faeces	
	Free %	Total %	Free %	Total %	Free %	Total %	Free %	Total %	Free %	Total %	Free %	Total %
1	39.7	53.4	6.7	11.2	0.4	0.6	46.8	65.2	26.0	31.5[1])	72.8	96.7
2	41.8	54.8	7.0	11.6	0.7	1.5	49.3	67.9	30.7	33.7	80.2	101.6
3	23.6	37.4	2.6	5.3	0	0	26.2	42.7	47.0	54.4[1])	73.2	97.1
4	24.5	37.0	6.7	11.3	0	0	31.2	48.3	43.8	49.8	75.0	98.1
5	37.9	53.3	0.8	3.9	0	0	38.7	57.2	27.0	30.3	65.7	87.5
6	45.8	54.4	4.4	7.7	0	0	50.2	6[illegible].1	28.4	33.0	78.6	95.1
7	33.7	50.4	0	2.0	0	0	33.7	52.5	32.4	34.8	66.3	87.3
Mean	35.3	48.7	4.0	7.6	0.2	0.3	39.5	56.6	33.6	38.2	73.1	94.8
s.e.m.	±3.2	±3.0	±1.1	±1.5	±0.1	±0.2	±3.6	±3.5	±3.2	±3.7	±2.1	±2.0

[1]) sulphanilic acid present from 48–96 hour faeces.

Table 6

Excretion of p-phenylenediamine sulphonic acid in the urine and faeces of rats after oral dosage with 100 mg Acid Yellow

Rat n	0–24 hour urine		24–48 hour urine		Total urine		0–48 hour faeces		Total urine and faeces	
	Free %	Total %	Free	Total %	Free %	Total %	Free %	Total %	Free %	Total %
1	–[1]		–[1]		–		30.9	42.3	–	–
2	4.3	5.4	0.6	0.8	4.9	6.2	33.7	48.8	38.6	55.0
3	1.9	3.2	0	0.8	1.9	4.0	39.3	58.5	41.2	62.5
4	1.9	2.8	0	0.8	1.9	3.6	27.1	54.5	29.0	58.1
5	3.5	4.8	0	0.4	3.5	5.2	29.8	42.5	33.3	47.7
6	4.8	7.1	0.9	1.3	5.7	8.4	46.1	49.9	51.8	58.3
7	4.1	5.9	0.2	0.7	4.3	6.6	42.8	47.7	47.1	54.3
Mean	3.4	4.9	0.3	0.8	3.7	5.7[2]	35.7	49.2	40.2[3]	56.0[3]
s.e.m.	±0.5	±0.7	±0.15	±0.12	±0.6	±0.7	±2.7	±2.2	±3.5	±2.0

[1] not estimated
[2] p-phenylenediamine sulphonic acid absent from 48–72 hour urines in animals 2–7
[3] mean values of animals 2–7

between 48 and 96 hours, and traces of sulphanilic acid were occasionally found in the 48–72 hour urines.

The mean recovery of sulphanilic acid in the acid hydrolyzed urine and the faeces was nearly 95%; together with the unchanged dye, this accounts for approximately 96% of the dose. Both urine and faeces gave higher recoveries of sulphanilic acid after acid hydrolysis, and the bound form was assumed to be the N-acetyl derivative. This matter was further examined by concentrating on a rotary evaporator a 24 hour urine sample after removal of salts by the addition of ethanol. The concentrate was streaked on cellulose plates, and N-acetylsulphanilic acid was added to the end of the band as a marker. After development in solvent system D this area was sprayed with Ehrlich's reagent to locate the marker and the adjacent area was scraped off the plate. The powder was eluted with aqueous ethanol, chromatographed again and eluted with water. The eluate was found to give the characteristic fluorescence properties of sulphanilic acid (table 2) after but not before acid hydrolysis.

The excretion of p-phenylenediamine sulphonic acid, the other amine arising from reduction of Acid Yellow, is shown in table 6. The mean recovery of this compound was only 56%, of which about 10% was found in the urine. As the samples showed increased amounts of p-phenylene diamine sulphonic acid after acid hydrolysis, the presence of an acetyl

derivative of this amine was also suspected. Chromatograms of both urine and faeces samples showed the presence of a substance having Rf values and giving a colour with Ehrlich's reagent identical with those of authentic 5-acetylamino-2-aminobenzene sulphonic acid. Further evidence that the metabolite was acetylated on the amino group meta and not ortho to the sulphonic acid group was obtained by its purification from faecal extracts. Further concentration and removal of interfering matter from the faecal extract was obtained by alternate evaporation on a rotary evaporator and addition of acetone The concentrate was chromatographed twice (Whatman no 1 paper), and the final-eluate was diluted with pH 8 phosphate buffer This solution showed an ultraviolet absorption maximum at 257 mμ and excitation and fluorescence maxima at 310 and 387 mμ, respectively These values correspond well with those for 5-acetylamino-2-aminobenzene sulphonic acid (table 2)

The acetylation of the absorbed amino sulphonic acids was studied in a group of three rats given 11.5 mg sulphanilic acid by intraperitoneal injection. The 24 hour urine samples contained 85 / of the dose as free sulphanilic acid and 13 / as the N-acetyl derivative No sulphanilic acid was found in either the 48 hour urine or the 0–48 hour faeces. Another group of three rats given 5 mg p-phenylenediamine sulphonic acid intraperitoneally showed the presence both of unchanged substance and 5-acetylamino-2-aminobenzene sulphonic acid in the 24 hour urine samples.

In order to obtain further evidence for the gastrointestinal origin of the reduction products of Acid Yellow two series of rats were given oral doses of sulphanilic acid and p-phenylenediamine sulphonic acid equal to the amount of these substances that would be formed from the complete reduction of 100 mg of the dye Table 7 shows that the excretion of sulphanilic acid is similar whether it is administered as such or derived from the dye (table 5) Both urinary and faecal excretions are similar and perhaps the only noteworthy difference is the finding that the excretion is somewhat more prolonged when Acid Yellow is given. The results from administering p-phenylenediamine sulphonic acid are shown in table 8 When compared with those in table 6, they indicate that faecal excretion is the same, regardless of the substance given, whereas the urinary excretion is slightly more than three times as high after dosage with p-phenylenediamine sulphonic acid This higher value is reflected in the increased 24 hour excretion values, which in one rat accounted for a third of the dose.

Discussion

Our results show that Acid Yellow when administered orally to rats, is extensively metabolized and that only about 1 / of the dose is recovered

Table 7

Excretion of sulphanilic acid in the urine and faeces of rats after oral dosage with 28.5 mg of sulphanilic acid.

Rat no	0–24 hour urine Free %	0–24 hour urine Total %	24–28 hour urine Free %	24–28 hour urine Total %	Total urine Free %	Total urine Total %	0–48 hour faeces Free %	0–48 hour faeces Total %	Total 0–48 hour urine and faeces Free %	Total 0–48 hour urine and faeces Total %
1	39 6	49.9	0.4	0.8	40.0	50.7	37.2	42.7	77.2	93.4
2	41 1	53 0	1.2	3 0	42.3	56.0	33 4	38 6	75 7	94.6
3	41 0	52.9	1.3	3.0	42.3	55 9	25 3	41.8	67 6	97 8
4	44.2	60.3	0.5	3 7	44 7	64 0	24.6	34 4	69.3	98.4
5	27.0	38.6	0.3	1 4	27.3	40.0	35 0	50.2	62.3	90.2
Mean	38.6	50.9	0.7	2.4	39.3	53.3	31 1	41 6	70.4	94.9
s.e.m.	±3 0	±3 5	±0 2	±0.5	±3 1	±3 9	±2.6	±2.6	±2.7	±1.5

Table 8

Excretion of p-phenylenediamine sulphonic acid in the urine and faeces of rats after oral *dosage with 36.9 mg of p-phenylenediamine sulphonic acid dihydrate.*

Rat no	0–24 hour urine Free %	0–24 hour urine Total %	24–48 hour urine Free %	24–48 hour urine Total %	Total urine Free %	Total urine Total %	0–48 hour faeces Free %	0–48 hour faeces Total %	Total 0–48 hour urine and faeces Free %	Total 0–48 hour urine and faeces Total %
1	20.6	22.2	0.3	0.8	20 9	23.0	27 1	48.7	48.0	71 7
2	29.0	33 4	0.3	1.6	29.3	35.0	27 1	32 6	56 4	67 6
3	5 1	6.8	0	0.6	5 1	7 4	33 4	52 9	38.5	60 3
4	16.7	19 6	0.2	0.5	16.9	20.1	43.3	47 9	60 2	68.0
5	5 3	7 0	0	0.3	5.3	7 3	43 1	64.9	48 4	72.2
Mean	15.3	17 8	0.2	0.8	15.5	18.6	34.8	49 4	50.3	68 0
s.e.m	±4 6	5.0	±0.07	±0 2	±4 6	5.2	±3 6	±5.2	±3 8	±2.1

unchanged in the urine. On the basis of the theory of gastrointestinal absorption developed by Brodie and co-workers (Brodie 1964), *little or no absorption of this substance would be expected, owing to the presence* of two highly ionized sulphonic acid groups. However Acid Yellow is readily reduced to its component sulphonic acid amines by the flora of the gastrointestinal tract, and there is therefore a competition between its reduction and its absorption Some *preliminary* results show that both *phosphate buffer extracts of rat faecal* contents and *Enterococcus sp* isolated from rat faeces can readily reduce Acid Yellow with the concomitant

appearance of sulphanilic acid and p-phenylenediamine sulphonic acid (LONGBERG & SCHELINE, unpublished). It is possible that a reduction in activity of the intestinal bacteria would result in increased Acid Yellow absorption.

Other factors may also enter here, and it is to be noted that the rats in which the common bile duct was cannulated consistently excreted more dye in the urine The reason for this is not known, but the dye excretion was prolonged in these animals, and this may reflect a reduced motility of the gastrointestinal tract, resulting in slower passage of the dye Also, the absence of bile from the intestines of these rats may influence the absorption of Acid Yellow

The absorption of the two sulphonic acids arising from reduction of Acid Yellow was considerably greater than that of the parent compound. Sulphanilic acid has a pKa of 0.988 and will be in effect completely ionized in the intestinal lumen. Nevertheless, it was found in the urine to the extent of about 40 % as such and 15 % in a conjugated form, after dosing either with the dye or with sulphanilic acid itself The absorption of p-phenylenediamine sulphonic acid was considerably less than this, but approximately 19 % of the dose was found in the urine after its administration. This lower value may be attributed in part to the fact that the substance is unstable in aqueous solution.

These results are in agreement with those obtained by RADOMSKI & MELLINGER (1962) who found that 1-amino-4-naphthalene sulphonic acid, one of the constituent sulphonic acids of Amaranth, was excreted to the extent of 18 % in the urine of rats after oral dosage. Other reports about the metabolism of the sulphonated azo dyes (DANIEL 1962 JONES *et al* 1964) describe this extensive and sometimes quantitative excretion of the constituent sulphonic acids after oral dosage. This must be considered to be the most striking feature in the handling of these substances by the body and is a good example of the effect of the normal intestinal flora on foreign organic compounds.

This subject appears to be relatively unexplored but a few recent publications indicate that the reaction of aromatic dehydroxylation also falls into this category SHAW *et al* (1961) reported that the m-hydroxy phenyl acids found in the urine of human subjects and rats after oral dosage with caffeic acid (3,4-dihydroxycinnamic acid) were absent when neomycin was given before the caffeic acid they believed that this dehydroxylation reaction was carried out by intestinal micro-organisms BOOTH & WILLIAMS (1963a & b) showed that dehydroxylation of catechol acids can be carried out by the intestinal contents of many animal species, and KAIHARA & PRICE (1963) found 8-hydroxyquinaldic acid to be the major urinary metabolite in rabbits given xanthurenic acid (4 8-dihydroxy-

quinaldic acid) orally Apart from the *in vitro* reduction of Acid Yellow noted above, saline or phosphate buffer extracts of rat faeces are also capable of forming 5-acetylamino-2-aminobenzene sulphonic acid when incubated with the dye (LONGBERG & SCHELINE, unpublished). It is conceivable that the effects of the intestinal flora on the metabolism of foreign organic compounds is of wider significance than generally realized The metabolic picture may be further complicated if these effects supplement those of enterohepatic circulation of the compound or its metabolites.

Our results indicate that biliary excretion is not an important route for the excretion of absorbed Acid Yellow Only about 5 % of the absorbed dye was found in the bile, a value in reasonable agreement with the results of RYAN & WRIGHT (1961) They found that approximately 10 % of an intravenous dose of Acid Yellow was excreted in the bile Thus urinary excretion is favoured over biliary excretion, and it would be expected that an intravenous dose, which gives a high blood concentration at one time, results in a higher biliary excretion than that after the more prolonged absorption associated with oral dosage

SMITH & WILLIAMS (1948) reported that sulphanilic acid is excreted unchanged in the urine by rabbits after oral dosage However DANIEL (1962) found that the 48 hour urines of rabbits given oral doses of the azo dyes Orange II Tartrazine and Sunset Yellow contained 49 74 and 54 % respectively of sulphanilic acid and 21 22 and 23 % respectively of N-acetylsulphanilic acid. The latter findings were based on chromatographic evidence and on the increase in colour given by the coupling reaction of BRATTON & MARSHALL (1939) after acid hydrolysis of the urines. RADOMSKI & MELLINGER (1962) reported the presence of sulphanilic acid but not its N acetyl derivative in the urine of rats after administration of Sunset Yellow Their evidence was obtained from chromatograms observed under short wave and long wave ultraviolet light. It is possible, however that the non-fluorescent N acetylsulphanilic acid was not detected in the presence of the many absorbing and fluorescing substances normally present in urine JONES *et al* (1964) found increased amounts of sulphanilic acid in the acid hydrolysed urines of the rat, rabbit and man after ingestion of Tartrazine. They assumed this to be due to the N acetyl derivative, but they did not identify it.

As the presence of N acetyl sulphanilic acid was also suggested by our experiments this matter was pursued further Chromatography of the urines with subsequent colour development gave equivocal results, because the yellow colour was slow to develop and was weak compared with that of the other metabolites. Also chromatograms of urine tend to give more diffuse spots, so that the presence of other metabolites, and

especially of urea, made the interpretation difficult. The N-acetylsulphanilic acid areas on the chromatograms after two developments and elutions were examined, and the fluorescence characteristics were determined before and after acid hydrolysis. The appearance of sulphanilic acid fluorescence after hydrolysis strongly suggests that the substance is N-acetylsulphanilic acid.

A similar acetylation was shown to occur with p-phenylenediamine sulphonic acid. Purification of this conjugate from faeces leaves no doubt that this acetylation occurred on the amino group meta to the sulphonic acid group as Rf values, colour reaction, ultraviolet absorption maximum and fluorescence characteristics agreed with those of authentic 5-acetyl amino-2-aminobenzene sulphonic acid.

It appears that these acetylated derivatives are formed both in the gastrointestinal tract and in the body after absorption of the amino sulphonic acids. The acetylated compounds found in the faeces must be of intestinal origin, because they are absent from the bile, a finding that rules out an enterohepatic cycle whereby the absorbed amino sulphonic acids could be acetylated and then excreted into the intestine. That the acetylation also occurs in the body is shown by the results obtained when *sulphanilic acid or p-phenylenediamine sulphonic acid was given intra* peritoneally. The N-acetyl derivative of each substance was found in the urine. It is not known whether this is the sole source of these urinary metabolites, as it is possible that absorption of some of the intestinal N-acetyl product may occur.

Summary

The absorption, metabolism and excretion of the sulphonated azo dye Acid Yellow was studied in rats after its oral administration.

About 1 % of the dose was found unchanged in the urine, and no dye was present in the faeces. Only about 5 % of the absorbed dye was excreted in the bile, and no reduction of the azo linkage of the absorbed dye occurred. Intraperitoneal injection of Acid Yellow resulted in rapid urinary excretion of the dye, but of none of its metabolites.

Acid Yellow was almost quantitatively reduced by the flora of the intestinal tract, and the resulting sulphanilic acid and p-phenylenediamine sulphonic acid were found in the urine and faeces. Appreciable gastrointestinal absorption of these sulphonic acids occurred. After Acid Yellow administration, totals of 57 % and 6 % of the dose were found in the urine as sulphanilic acid and p-phenylenediamine sulphonic acid, respectively. When the amino sulphonic acids themselves were given, the corresponding values were 53 % and 19 %.

Conjugated forms of these two metabolites of Acid Yellow were found in urine and faeces and shown to be N-acetylsulphanilic acid and 5-acetylamino-2-aminobenzene sulphonic acid, respectively. These are formed both in the intestine and in the body after absorption of the amino sulphonic acids.

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p-Hydroxyphenylacetic Acid, β-Phenylethylamine and Tyramine in Post Mortem Livers Stored under Different Conditions

By

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It is well-known that analyses of autopsy material for poisons often reveals the presence of interfering substances. Such substances may have been formed, for example, during the extraction (Marquis 1896) or by post mortem processes (autolytic or bacterial or both)

The object of our investigation was to study the occurrence of three such substances in human liver tissue, with relation to the circumstances in which the tissue had been stored.

The three substances tyramine, p-hydroxyphenylacetic acid and β-phenylethylamine, have all been demonstrated previously in autopsy material.

As tyrosine may be thought more or less responsible for the formation of tyramine, p-hydroxyphenylacetic acid, and other p-substituted phenols (Thierfelder 1924) determinations of the contents of free and of free plus bound tyrosine were included.

Material and Methods

The material analysed consisted of liver tissue obtained from 109 legal autopsies. Of the 109 specimens, 17 were taken from persons dying during the warm season (June 10–Sept. 11) specimens originated from individuals dying from widely different causes. About 10% had died of poisoning, most of them with narcotics.

The specimens to be studied were treated, as far as possible, in conformity with the procedure usually employed for forensic specimens. They were stored in cleansed, but unsterilised, glass-stoppered glass tubes and left in the refrigerator and freezing cabinet generally employed for such specimens.

The state of the liver tissue as well as the temperature and place of storage varied. Some specimens (106) of 50–100 grams each were stored at 4–5 These had been taken from as many individuals submitted to legal autopsy up to 4 days after death. Seven of these speci-

mens were minced in a meat chopper before being stored. The remaining 99 were not minced until the day of analysis. The amount left after the first analysis of minced tissue from 20 specimens was divided into two equal groups, which were stored at 4–5° or −18° until analysed again.

Five of the specimens stored at 4–5° were analysed three times, the last time after storage for 526 days.

Three specimens were taken on autopsies carried out 5, 17 or 27 days after death.

Extraction

Liver tissue, 5 g, was homogenised with 5 ml of water and the pH of the homogenate was determined with a glass electrode. Water was added to 20 ml, and the mixture was deproteinised by adding 5.0 ml of 30 % (w/v) trichloracetic acid and centrifugation after standing for 10 minutes. A measured portion of the supernatant fluid was rendered 0.5 N in hydrochloric acid and boiled under reflux for 60 minutes. This caused the trichloracetic acid to be destroyed. After cooling down, the contents were extracted three times, each time with an equal volume of ether ( ), and then another three times, each time with an equal volume of chloroform at alkaline reaction, i. e. at pH above 1 (*b*). Saturated sodium borate solution, 2 ml, were added, together with solid sodium chloride in excess. The pH was adjusted to about 9.5 by means of 2 N HCl. The mixture was extracted four times (*c*), each time with equal volume of chloroform-isopropanol (3 + 1 by vol.). The four substances were separated by the process of extraction, p-hydroxyphenylacetic acid being found in the ether phase (*a*), β-phenylethylamine in the chloroform phase (*b*), tyramine in the chloroform-isopropanol phase ( ), tyrosine remaining in the aqueous phase (*d*). Anhydrous sodium sulphate was added to the non-aqueous extracts, which were submitted to filtration and evaporation to dryness, 200 µl of conc. HCl having been added to the chloroform phase (*b*), to convert the volatile β-phenylethylamine into the corresponding hydrochloride before evaporation.

Total tyrosine content (free and bound)

A weighed amount (40–50 mg) of liver homogenate (1 + 3 w/w) was mixed with 2000 µl of 6 N-HCl and submitted to acid hydrolysis in sealed glass tubes in a thermostat at 113° for 4 hours (hydrolysis time of 4 to 6 hours has been found to be optimal). The hydrolysate was then evaporated to dryness in an open porcelain dish on a boiling water bath to remove the hydrochloric acid. The residue was dissolved in 40.0 ml of water and this solution was used for determining tyrosine.

By the procedure described tyramine was included in the determination. The two substances can be separated by thin layer chromatography of the residue after evaporation, obtained by system B, as described in table I.

Thin layer chromatography

Thin layer chromatography was performed on 20 × 20 cm plates with 0.5 mm layer of silica gel-G activated by heating at 110° for one hour. The plates were stored in desiccator over silica gel.

Solutions for development and localisation have been set out in table I. The residue to be chromatographed was dissolved in methanol. Two measured portions of the methanol solution were transferred to one section each. To the methanol solution containing residue from the ether phase (*a*) was added conc. aqueous ammonia to neutralise any unconverted

trichloracetic acid. Excess of NH_3 was then removed by evaporation before pplication. After development, one section of the dry chromatogram was localized (the other was covered all the while. Sections containing β-phenylethylamine were sprayed before covering with conc. HCl to bind it) The area the covered section correspond g to the localized

Table 1

Thin layer chromatography

R_F values, colour reactions (in brackets fluorescence under 350 mμ), and sensitivity (μg/cm²) for some of the substances that may occur the residues of the organic extracts *a*, *b* and *d*

Solvent systems.

System A Isopropanol, water and ethyl acetate 24 11 65 (ST ML & KALTENBACH 1961). The chromatogram saturated for 20 minutes nd developed for 50 minutes.

System B Chloroform nd diethylamine 46 4 (freshly mixed) Vessel and vessel plus plate saturated for 30 and 10 minutes, respectively with development for 10 minutes.

System C Butanol, glacial acetic acid, and water 40 10 50 (PARTRIDGE & WESTALL 1948) The chromatogram saturated for 30 minutes (lower phase) nd developed for 90 minutes (upper phase).

System D Conc. NH_3, water nd butanol 5 10 85. The chromatogram saturated for 20 minutes nd developed for 40 minutes.

Reagents

Reagent 1 0.1% 1-nitroso-2 naphthol I ethanol, and 2 N HNO_3 with 0.1 g $NaNO_2$ added per 10 ml. The plate heated to about 60° (GERNGROSS, VOSS & HERFELD 1933)

Reagent 2 0.5% fresh diazotized p-nitroaniline. The plate sprayed with 2 N-N $_2CO_3$ after about 4 hours (WICKSTRØM & SALVESEN 1952)

Substance	Resi-due	R_F in solvent system				Rea-gent	Colou (fl oresc.)	Sensitivity (μg/cm²)
		A	B	C	D			
p-hydroxyphenyl-acetic acid		0.28				1	red (light yellow)	0.3–0.4
p-hydroxyphenyl-propionic acid		0 44	–			1	red (reddish yellow	0.1–0.2
p-hydroxyphenyl-ethanol		0.93				1	red (light yellow)	0.–0.3
p-hydroxybenzoic acid	a	0.54				1	red	–
p-hydroxybenz aldehyde						1	0 (0)	
β-phenylethylamine	b		0.42	–		2	red	1.0–1.5
tryptamine	b	–	0.12		–	2	yellow	1.0–1.5
tyramine				0.55	0.27	1	red (yellow)	0.–0.3
tyrosine	d			–	0 07	1	red (yellow)	–

substance was scraped off and the scrapings were eluted by 5.0 ml of 70 % ethanol (96 % ethanol for β-phenylethylamine) with magnetic stirring for 15 minutes. The extract was centrifuged, and measured portions were taken for determining the different substances.

Quantitative determinations

Tyrosine, tyramine and *p-hydroxyphenylacetic acid* were determined fluorometrically by a method based on the fact that various para substituted phenols react with 1-nitroso-2-naphthol and N NO_2 in HNO_3 to give a fluorescent derivative. The method is a modification of one described by WAALKES & UDENFRIEND (1957) for determining tyrosine in plasma and tissues.

Reagent: 1-nitroso-2-naphthol 0.1 %: 1-nitroso-2-naphthol in a mixture of equal volumes of 96 % ethanol and 0.1 N-HCl.

Nitrite-R: 24.5 ml of 1 : 5 HNO_3 (v/v) mixed with 0.5 ml of freshly prepared 2.5 % $NaNO_2$.

Procedure: Measured portions of the eluates from the chromatographic purification of the ether phase ( ) and the chloroform-isopropanol phase ( ), as well as 50 µl of the aqueous phase (*d*), were diluted each to 2 ml with water or ethanol to give an alcohol concentration of 50 %. Then 1 ml of 1-nitroso-2-naphthol R and 5 minutes later 1 ml of nitrite-R were added. The tube was stoppered and placed in a bath of 55° for 30 minutes. After cooling down, 4 ml of chloroform and 6 ml of water were added. The tube was shaken and centrifuged to remove excess of 1-nitroso-2-naphthol from the aqueous phase. The aqueous phase was transferred to a cuvette and measured in a Coleman electronic photofluorometer model 12B. The mercury line at 436 mµ (filter OB1) was used as primary light, and as secondary filter OY 4, which is impermeable to light of wavelengths under 490 mµ.

For determination of p-hydroxyphenylacetic acid excess of 1-nitroso-2-naphthol was removed from the derivative produced, by extraction with chloroform at pH 7.7 obtained by adding 1 ml of 3 M K_2HPO_4 and about 300 µl of 8 N-NaOH. A reagent blank was employed for adjustment of the galvanometer zero. The apparatus used allowed determination of 0.05 µg/ml, and there was proportionality between concentration and fluorescence for concentrations up to at least 0.4 µg/ml in the final solution.

The compound p-hydroxyphenylpropionic acid, also noticed in autopsy material, interferes with the determination of p-hydroxyphenylacetic acid, the propionic acid derivative fluorescing with the same intensity as the acetic acid derivative. The two substances can, however, be separated by thin layer chromatography system A (table 1). p-Hydroxyphenylethanol and p-hydroxybenzaldehyde may also both be present in autopsy material. The derivative produced from p-hydroxyphenylethanol will fluoresce with the same intensity as the corresponding derivative from p-hydroxyphenylacetic acid. However, no more than the p-hydroxybenzaldehyde derivative, will interfere with the determination, these being both soluble in chloroform and therefore removed with the excess of 1-nitroso-naphthol.

β-Phenylethylamine was determined by a procedure employed by DILL *et al.* (1956) for determining 5,5-diphenylhydantoin. The principle of the method is that of nitrating the phenyl nucleus of β-phenylethylamine and reducing the nitro group to a primary aromatic amine, which is determined by diazotisation and coupling, with centrifugation and measurement of absorption at 555 mµ after standing for 4 hours.

A reagent blank was also examined.

By the method described, 0.5 µg/ml gave an extinction of about 0.14 for a layer of 1 cm in thickness. There was proportionality between concentration and extinction for concentrations up to at least 5 µg/ml in the solution measured, for which reason only one standard value was included.

Tryptamine, also found in autopsy material, will interfere with the determination of

β-phenylethylamine, but the substances can be separated by thin layer chromatography system B.

A more direct method of determining β-phenylethylamine, as a diazo-amino compound from coupling with diazotised p-nitroaniline has been developed by BEYER & SKINNER (1940) and modified by ALLES & WISEGARVER (1961). This method could not be used by us, however because diethylamine from the chromatographic purification uses up the reagent, so that the red colour characteristic f primary amines (ALLES & WISEGARVER 1961) cannot be reproduced quantitatively

Recovery experiments

The experiments were made on human liver tissue from a person killed in a road accident. The liver had been stored at −18 for 6 months. It displayed no signs of putrefaction.

The liver tissue was homogenised with an equal weight of water. Solutions of tyrosine, tyramine, β-phenylethylamine o p-hydroxyphenylacetic acid were added to specimens of 10 g each, in amounts of 0, 10, 50, and 500 µg per gram liver tissue. Equal quantities of all four substances were dded to each specimen of liver homogenate. After the solution had

Table 2

Recovery by quantitative determination of tyrosine, tyramine, p-hydroxyphenylacetic acid and β-phenylethylamine in two specimens of the same human liver analysed at an I terval of 24 hours, after adding known quantities of tyrosine, tyramine, p-hydroxyphenylacetic acid and β-phenylethylamine. For tyrosine are recorded both th original content, called th genuine and the proportion of the added amount recovered termed "recovery"

Added µg per g liver tissue	0		10	50	500
	Genuine	Recovery	Recovery	Recovery	Recovery
Tyrosine	186	0	8	43	485
	262	0	10	53	487
Average.		0	9	48	486
(Recovery in per cent)		(0)	(90)	(95)	(97)
Tyramine		0	8.7	37.5	384
		0	8.8	37.4	395
Average.		0	8.7	37.5	391
(Recovery I per cent)		(0)	(87)	(75)	(78)
p-hydroxyphenylacetic acid		0	7.7	38.9	445
		0	8.5	38.9	463
Average		0	8.1	38.9	457
(Recovery in per cent)		(0)	(81)	(77)	(91)
β-phenylethylamine		0	6.0	40.7	466
		0	7.3	38.2	463
Average			6.7	39.5	465
(Recovery in per cent)		(0)	(67)	(79)	(93)

been added, the mixture was diluted with water t a total volume of 20 ml, and analyses were then performed as described above, determinations being carried out in duplicate.

The results of the recovery experiments are recorded in table _, which shows that from 67 to 97 of the added amounts were recovered. This percentage is considered satisfactory for the purpose.

Results

Tables 3 4 and 5 illustrate the occurrence of tyramine, p-hydroxy phenylacetic acid and β-phenylethylamine, respectively in liver tissue.

Amounts of less than 3 μg substance per gram liver were too small to be detectable by fluorometry or colorimetry the amount present was then estimated on the basis of the colour reaction seen after spraying the plate chromatogram.

The analyses described above gave the results that the contents of tyramine, p-hydroxyphenylacetic acid and β-phenylethylamine were

Table 3

Range of tyramine contents in liver tissue (μg/g), classified according to period nd temper ture of storage. The total number of specimens i the group nd the number of positive specimens as a percentage of the total umber are given. The period of storage af 4–5 is reckoned from the day of death. The period of storage at −18 is reckoned from the day of freezing. Nothing demonstrated on day of freezing.

Days of storage	Number of specimens (<0.25)	positive 0.25–2.9	3.0–9.9	10–200	>200	Total of specimens	Percentage positive
4–5							
0–4	20					20	0
5–13	39	6				45	13
14–20	13		4		1	20	35
21 30	6		3	6	2	17	59
31–49		–		3		7	100
50–120				4	4	10	100
526 (about 18 mths.)			2	2	1	5	100
−18							
about 30						2	0
480 (about 15 mths.)	6					8	25

) Below limit of demonstration.

independent of the state of the tissue (whole or minced) and of the place where the organ was kept (in the cadaver or in a glass tube at 4–5°) The concentrations were not much higher in the specimens originating from individuals dying during the warm season (June 10 to Sept. 11) than in the others.

Hence, none of these factors have been considered in the tables. Table 3 shows that *tyramine* was not demonstrated in liver tissue left for maximum of 4 days after death in the body (including the period in *la Morgue*) or in glass tubes at 4–5° Tyramine was noticed in some of the specimens stored for more than 4 days in the circumstances described It is seen in the table that tyramine occurred more frequently and in higher concentrations after fairly long storage of the specimen. A content of 0.25–3 μg/g was seen in some specimens after 5 to 13 days Values ranging from 3 to 10 μg/g have not been noticed until after storage for 14 days, but a content exceeding 200 μg/g may be found after the same period of storage

The analyses also demonstrated that the content of tyramine in liver tissue stored in a minced state at 4–5° for about 18 months constituted only on an average 10 / of that seen after storage for about 80 days. This tendency is illustrated in table 3

Table 3 shows further that tyramine was detected in all the specimens stored for more than 30 days at 4–5 but in none of those stored for the same period at −18°

After storage at −18° for 18 months tyramine was found in 25 / of the specimens analysed, but at low concentrations (0 4 μg/g).

As shown in table 4 *p-hydroxyphenylacetic acid*, unlike tyramine, was found in concentrations of up to 10 μg/g in 75 / of the specimens stored for a maximum of 4 days after death in bodies or glass tubes at 4–5° though the limit of demonstration is higher for p-hydroxyphenylacetic acid than for tyramine. A concentration of 10 μg/g was noticed in some specimens stored for 5 days. Over 200 μg/g was found in a single specimen only after storage for 50 days.

Further the analysis of liver tissue stored in a minced state at 4–5 for about 18 months showed that the content of p-hydroxyphenylacetic acid unlike that of tyramine, increased continuously during this period This is not clearly shown in the table.

It is also shown in table 4 that the probability of finding p-hydroxy phenylacetic acid in tissue stored for more than 26 days in the circum stances described is 96.5 %.

Of the tissue specimens frozen down to −18 those stored for 30 days were found to contain no p-hydroxyphenylacetic acid, whereas 75 % of the specimens stored for 18 months contained this substance, though at low concentrations (under 3 μg/g)

Table 4

Range of p-hydroxyphenylacetic acid contents in liver tissue (µg/g), classified according to period and temperature of storage. The total number of specimens in the group and the number of positive specimens as a percentage of the total number are given. The period of storage at 4–5 is reckoned from the day of death. The period of storage at −18° is reckoned from the day of freezing. Nothing demonstrated on day of freezing.

Days of storage	Number of specimens					Total of specimens	Percentage positive
	(<0.35*)	positive					
		0.35–2.9	3.0–9.9	10–200	>200		
4–5							
0–4	5	13	2	–		20	75
5–10	11	21	2	1		35	69
11–26	8	23	6	4		41	80
27–49	–	5	2	6		13	100
50–120	1	–	1	7	1	10	90
526 (about 18 mths.)	–	–		5	–	5	100
−18°							
about 30	2						0
480 (about 15 mths.)	2	6		–	–	8	75

*) Below limit of demonstration.

In table 5 it is shown that *β-phenylethylamine* was not demonstrated in liver tissue stored for a maximum of 4 days after death in bodies or glass tubes at 4–5° it must be pointed out that the limit of demonstration is considerably higher for β-phenylethylamine than for tyramine and p-hydroxyphenylacetic acid.

After storage for more than 4 days the presence of β-phenylethylamine was observed, but only sporadically A concentration of 3 µg/g was noticed in some specimens after 9 days, but values exceeding 10 µg/g did not occur until after storage for 23 days. Values above 200 µg/g were not observed even after storage for 120 days.

The table shows further that β-phenylethylamine was not detectable in liver tissue stored at −18 for 30 days or 18 months.

It is worth mentioning that by spraying diazotised p-nitro-aniline on the plate chromatogram with the *b* residue applied which may contain β-phenylethylamine, an intense red colour reaction would often occur from specimens taken soon after death within the area near the R_F value of about 0 02.

Table 5

Range f β-phenylethylamine contents in liver tissue (μg/g) classified according to period and temperature of storage The total umber of specimens in the group and the umber of positive specimens as a percentage of the total umber are given. The period of storage at 4–5 is reckoned from the day of death. The period of storage at −18 is reckoned from the day of freezing Nothing demonstrated on day of freezing.

Days of storage	Number of specimens					Total of specimens	Percentage positive
	(<1.5)	positive					
		1.5–2.9	3.0–9.9	10–200	>200		
4–5°							
0–4	20	–		–	–	20	0
5–8	26	1		–	–	27	4
9–22	41	–	1	–	–	42	2
23–49	16		1	3	–	20	20
50–120	7	–	1	2	0	10	30
−18°							
about 30	2	–			–	2	0
480 (about 15 mths)	8	–	–	–	–	8	0

) Below limit of demonstration.

The analyses for free and free plus bound tyrosine showed concentra tions of *free tyrosine* ranging from about 70 to 3500 μg/g, generally higher the longer the specimen had been stored, even at −18

The average concentration of *free plus bound tyrosine* was determined as 6 5 mg per gram liver tissue. The results were based on specimens in which no tyramine was demonstrated. After storage for 18 months at 4–5 an average concentration of 2.1 mg/g was found (from 1.2 to 3 7 mg/g)

The pH values ranged between 5 7 and 6 3 even in specimens stored for one month. Specimens stored at 4–5 for 18 months were found to have a pH of about 8.3 whereas the pH remained unchanged at −18

Summarising, the analyses gave the results that p-hydroxyphenylacetic acid was detected after storage for up to 4 days after death and that tyramine was sometimes demonstrable at a concentration exceeding 200 μg/g after storage for 14 days. The analyses also showed that formation of the interfering substances mentioned can be appreciably reduced by storing the tissue at −18

The acids seem to appear before the amines.

Finally it is worth noting that *tryptamine* which may interfere with the spectrophotometric estimation of ordinarily occurring alkaloids (KÆMPE 1965 b) was detected by the chromatographic purification procedure only *in the same specimens*, except in one in which β-phenylethylamine was observed. Similarly *p-hydroxyphenylethanol* which may interfere with the spectrophotometric estimation of 5 5-disubstituted barbituric acid derivatives (KÆMPE 1964 a) was only found by the chromatographic procedure in nearly all the specimens containing p-hydroxyphenylacetic acid On the other hand, a positive reaction was more rarely seen corresponding to the "pseudobarbituric acid" (JACKSON & FINKLE 1963) *p-hydroxyphenylpropionic acid*

Discussion

The substances mentioned above may interfere with quantitative determinations of certain poisons because of the similar conditions of their extraction.

Unless corrective measures are taken the polarographic determination of morphine (KÆMPE 1964 b) will involve tyramine, this having approximately the same half wave potential (−0 95 v) as morphine (−1 02 v). A content of 10 µg tyramine will then correspond to 1 7 µg morphine.

The absorption conditions at 240 mµ are the same for p-hydroxyphenylacetic acid as for 5 5-disubstituted barbituric acid compounds (KÆMPE 1965 a) Here 10 µg p-hydroxyphenylacetic acid corresponds to a content of 9 6 µg aprobarbital

On analysis for amphetamine by the method of ALLES & WISEGARVER (1961) 10 µg β-phenylethylamine will yield the same colour as 8 6 µg amphetamine. Further the spectra of β-phenylethylamine, amphetamine and pethidine in the ultraviolet region have many points of resemblance

In analysing autopsy material for the poisons mentioned, a separation is therefore required when such interfering substances occur

As mentioned previously (KÆMPE 1964 b & 1965 a), tyramine and morphine as well as p-hydroxyphenylacetic acid and ordinarily occurring barbituric acid compounds can be separated by chromatography among other methods.

β-Phenylethylamine can be separated from both amphetamine and pethidine, these two substances having R_F values of 0 55 and 0 66 respectively in system B (table 1)

The carrying capacity of the chromatograms used (Whatman no 1 or 0.25 mm thickness) is judged to be 100 µg substance for an application zone of 2 cm An application zone exceeding 2 cm is useless for analysis

of small amounts of poisons. A concentration over 100 μg of the interfering substances will thus disturb the purification procedure.

An amount over 100 μg will be present when the tissue analysed contained over 2.0 μg per gram and the extraction is carried out on 50 g tissue, the amount usually employed in this Department for direct extraction. For extraction by the so-called Stas-Otto method we use 300 g and an application zone of 12 cm Direct extraction for barbituric acid compounds is performed on no more than 10 g tissue. A content over 100 μg p-hydroxyphenylacetic acid will therefore not be attained below concentrations of 10 μg/g.

As the three substances mentioned above may occur at the disturbing concentrations described as soon as 5 days after death, if the organ remains in the body or is stored in the whole or minced state at 4 we may conclude that 1) organs to be submitted to chemico-legal analysis should be analysed immediately after the autopsy or if this is impossible, should be stored frozen. 2) Special measures must be taken (e. g. re-chromatography) when analysing tissue stored for more than 5 days at 4 or left in the cadaver

A corresponding analysis of gastric contents taken from legal autopsies must be expected to give different results, depending in some measure on the food consumed. For example, it may be mentioned that gastric contents will contain tyramine immediately after consumption of seasoned cheese and tryptamine after consumption of tomatos.

Summary

Analyses of post mortem liver tissues kept for different periods at different places and temperatures disclosed the presence of p-hydroxyphenylacetic acid in both "fresh" and stored material Tyramine and β-phenylethylamine were generally not detected until after 5 days storage It seemed not to matter whether the organ was stored in the whole or the minced state After storage of liver tissue for 18 months at 4–5 the concentration of tyramine was found to be lower than after 80 days, whereas that of p-hydroxyphenylacetic acid was higher

The formation of the three substances can be checked by storing the organ at −18 Tyrosine was liberated even during storage at −18

The significance is discussed of the post mortem formation of tyramine, p-hydroxyphenylacetic acid, and β-phenylethylamine and their effect on the reliability of the chemicolegal analyses for morphine, 5,5-substituted barbituric acid compounds and amphetamine or pethidine, respectively

Finally it is worth noting that *tryptamine* which may interfere with the spectrophotometric estimation of ordinarily occurring alkaloids (KÆMPE 1965b), was detected by the chromatographic purification procedure only in the same specimens, except in one, in which β-phenylethylamine was observed Similarly *p-hydroxyphenylethanol* which may interfere with the spectrophotometric estimation of 5,5-disubstituted barbituric acid derivatives (KÆMPE 1964a), was only found by the chromatographic procedure in nearly all the specimens containing p-hydroxyphenylacetic acid On the other hand a positive reaction was more rarely seen corresponding to the pseudobarbituric acid" (JACKSON & FINKLE 1963) *p-hydroxyphenylpropionic acid*

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Pentetrazol Induced Seizures and their Antagonism by Barbiturates in Newborn and Young Mice

By

H. Ferngren

(Received March 4 1965)

The influence of age on effect of drugs has in recent years received increasing interest from pharmacologists and pediatricians. The two main reasons for this are probably the discovery of the immaturity of certain drug-metabolizing enzyme systems at birth and reports of enhanced toxicity of some drugs in the neonatal period (for review see NYHAN 1961 and DONE 1964)

Because of the functional immaturity of the central nervous system in most mammals at birth, it would be expected that age would affect drug activity in the central nervous system. In fact various age-related differences in the effect of convulsants and anticonvulsants, as well as in the effect of cortisol on the seizure treshold, have been found by different workers (CADILHAC *et al* 1960 PYLKKÖ and WOODBURY 1961 KOBAYASHI *et al* 1963 VERNADAKIS and WOODBURY 1963 & 1964 VULPE *et al* 1963)

Our study was undertaken to ascertain the anticonvulsant effect in growing mice of two common drugs, phenobarbital (= phenemalum NFN) and mephobarbital (= enphenemalum NFN) Pentetrazol (= pentylenetetrazol) was selected as the seizure inducing drug, because it has been widely used for anticonvulsant tests and its pharmacology is well-known (reviewed by HAHN 1961) By determining the median convulsant and anticonvulsant doses at different ages it was hoped to obtain meaningful comparisons between ages and drugs

Materials and Method

The experiments were performed mice f the NMRI strain. About 75 young animals were used for each age group and drug. On the day after birth the nimals were received in litters together with their mothers. The you g animals were kept with their mothers until

Table I

Mean weight i grams of the total number of young mice for each age group used. The significance f the differences between ge groups (Student s t-test) is given in the table.

Age i days	n	mean ± s e m.	Comparison of age groups	p
1	839	1.58 ± 0.0075	1 3	<0.001
3	397	1.89 ± 0 0135	3–5	<0.001
5	703	2.45 ± 0.0179	5–7	<0.001
7	82	3.07 ± 0 0498	7–9	<0.001
9	460	4 02 ± 0 0785	9–12	<0.001
12	79	4.54 ± 0.0456	12 21	<0.001
1	84	8 17 ± 0 0477	21–adult	<0.001
dult males	477	18 6 ± 0 0772		

the time of study Each animal was used only once. The experiments were performed at an ambient temperature of 20 No attempt to separate the sexes was made. The mean weights of the d fferent ages and total numbers of animals used in this study are given i table I

Test solutions

1 *P ntetrazol.* A 10 (w/) solution 0 9 % (w/v) N Cl was used.

2. *Phenobarbital* = phenemalum NFN = (5-ethyl-5-phenylbarbituric acid). A 1 % (w/v) sol ti of the sodium salt in 0.9 N Cl was used for all ge groups except th 1-day old animals, which received a 0.2 % sol tion.

3 *Mephobarbital* = mephenemalum NFN = (5-ethyl-1 methyl-5-phenylbarbituric acid). A 1 g mount of the drug was dissolved in 4.06 ml 1.0 N NaOH and 40 g of propylene glycol were added during slow heating. The solution was made up to 100 ml with distilled water This 1 % (w/v) soluti was used for all groups except the 1 day old nimals, for which a 0.. % solution was used.

Method of injection

All injections were given subcut neously and dorsally w th Hamilton microsyringes conta ning (0 25 or 50 µl By using small olumes of drugs, w ter balance disturbances ere minimized. The injected volumes per animal varied between 2 and 9 µl for pentetrazol, 1 and 20 µl for phenobarb tal and 1 nd 28 µl for meph barbital depend g on weight.

Determination f CD50

The CD50 for each age group was defined as the dose at which 50 of the animal had seizure, either tonic or clonic in type but occurring within 60 minutes of pentetrazol injection and lasting for t least 5 seconds. In each age group of 75 animals 3 to 5 doses were given at logarithmic ntervals. The number of nimals with convulsions at each dose was recorded. The CD50 at 95 fiducial limits ere calculated by probit nalysis (Ph. Nord. vol. IV).

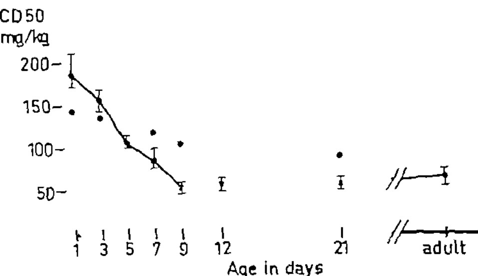


Fig. 1 Median convulsive doses (mg/kg for pentetrazol (●) injected subcutaneously into growing mice of different ages. Theoretical CD50 (◐) calculated from the mean body area for each age group in relation to the median convulsive dose and mean body area for adult animals are included for comparison. Vertical bars indicate 95% fiducial limits.

Determination of ED50

The ED50 for each age group and anticonvulsant drug was defined as the dose that prevented convulsions in 50% of the animals when pentetrazol, at a dose 1.5 times the CD50, was injected at a given interval after the anticonvulsant. (For phenobarbital after 30, 50 or 90 minutes, for mephobarbital after 50 minutes). Both barbiturates were injected 3 to 5 groups of mice in each age group in logarithmically spaced doses. The number of mice with convulsions from each dose was recorded and the ED50 at 95% fiducial limits was calculated by probit analysis (Ph. Nord. vol. IV) using 3, 4 or 5 dosages.

Results

The CD50 of pentetrazol in mice of increasing age is shown in fig. 1. The convulsant dose decreases with advancing age until 9 days, when it falls to the same level as in adult mice.

As the body surface area is occasionally used in recommending doses for children, theoretical CD50 values were calculated on the basis of body surface area related to adult mice. The calculations were based on Dawson's formula

$$D = \left(\frac{W}{70}\right)^{e}$$

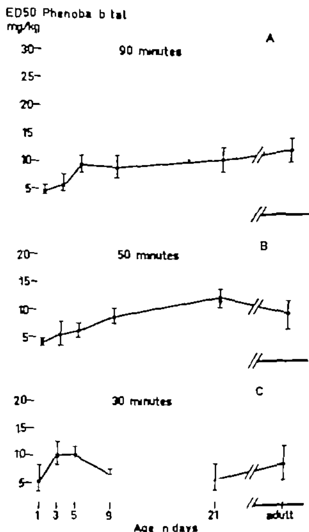


Fig. 2. Median anticonvulsant doses in mg/kg for phenobarbital (●) injected subcutaneously against pentetrazol-induced seizures in growing mice of different ages. The intervals between the injections of the two drugs are 90 minutes (A), 50 minutes (B) and 30 minutes (C). Theoretical ED50 values ( ) calculated from the mean body area for each age group and interval in relation to the median anticonvulsant dose and mean body area for adult animals are included for comparison. Vertical bars indicate 95 % fiducial limits.

with the symbols meaning

D = fraction of adult median dose,
W = mean weight of the age group, and
p = ? the mean weight of the adult mice
has been substituted for 70

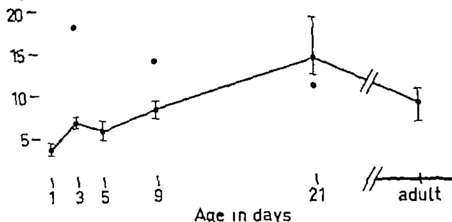


Fig. 3 Median anticonvulsant doses in mg/kg for mephobarbital (●) injected subcutaneously against pentetrazol-induced seizures in growing mice of different ages. The interval between the injections of the two drugs was 30 minutes. Theoretical ED50 values (◐) calculated from the mean body area for each age group in relation to the median anticonvulsant dose and mean body area for adult animals are included for comparison. Vertical bars indicate 95 % fiducial limits.

The calculated theoretical values have been incorporated in fig. 1 as semi-open circles for comparison with the values found experimentally

The type of convulsion also differed according to age. In the newborn body the colour became paler respiration ceased tonus of the body increased, urination occurred, and all 4 limbs were extended tonically with the hind paws twitching. With advancing age this picture changed to clonic-tonic convulsions with increasing clonic activity Urination did not occur in mice over 9 days of age

The median effective doses of phenobarbital against pentetrazol-induced seizures in newborn and young mice at different time intervals are given in fig. 2. This shows that the variation with age of the anticonvulsant effect of phenobarbital depends on the time after the injection of pentetrazol when the phenobarbital injection is made. It is only for newborn mice that the ED50 values are similar for all three intervals.

The theoretical ED50 values corresponding to adult mice dosage corrected for body surface area have also been calculated for phenobarbital. These values are included as semi-open circles in the figure. It can be seen that, regardless of the interval between the injections, the ED50 values for the younger animals are smaller than would be expected from their surface area.

Table 2

Induction time, in minutes, for seizures after subcutaneous i jection of pentetrazol into growing mice. The significance of the differences between age groups (Student t-test) is given i the table.

Age i days	Pentetrazol n	mean ± s.e.m.	Comparison of age groups	p
1	43	25 7 ± 1 4	1 3	<0.001
3	36	16.1 ± 1 6	3–5	0.1–0 05
5	50	20.8 ± 1 9	5–9	=0 05
9	37	15 2 ± 2.1	9 1	<0 001
21	35	3 3 ± 0.4	1–adult	<0.001
adult males	26	10.8 ± 0.9		

Table 3

Induction time, in minutes, for seizures after subcutaneous injectio of pentetrazol into growing mice injected 50 minutes previously with phenobarbital. The significance of the differences between ages groups (Student s t-test) is given in the table.

Age in days	Phenobarbital Pentetrazol n	mean _ s.e.m.	Comparison f age groups	p
1	25	20 2 _ 1 9	1 3	=0.6
3	2_	19 0 1 1	3–5	<0 01
5	35	25 3 _ 2.0	5–9	0 05–0 0
9	21	18.4 – 2.3	9–21	<0.001
21	25	4 6 ± 0 7	21–adult	<0.01
ad lt males	31	9.3 1.5		

The median effective dose of mephobarbital against pentetrazol-induced seizures in mice at different ages is given in fig. 3 with theoretical values calculated from body surface area included as semi-open circles. This curve closely resembles that of phenobarbital for the same time interval (50 minutes)

The mean induction time before the onset of convulsions after pentetrazol injected alone or following a previous injection of phenobarbital is given in table 2 and 3 for the different ages These tables shows that the mean induction time is relatively constant until after the age of 9 days,

when a fall occurs down to the minimum in 21 days old mice. These tables also show that previous injection of an anticonvulsant drug does not significantly affect the induction time.

Discussion

The variation with age in the convulsant activity (CD50) of pentetrazol has been studied by other workers. THEOPOLD & FÖRSTER (1956) injected a standard dose (mg/kg weight basis) intravenously into rabbits of various ages and found an increasing frequency of seizures with increasing age. CAHILHAC *et al* (1960) found in newborn kittens, in which cortical and deep EEG recordings were studied during barbiturate anaesthesia, that intraperitoneally injected pentetrazol evoked seizure activity mainly subcortically and that this activity was accompanied by motor phenomenona. On the other hand KOBAYASHI *et al* (1963) could not evoke seizures with pentetrazol injected intraperitoneally into newborn mice, even in doses as high as 400 mg/kg, but found what they call hyperkinesia. Changes in the cortical EEG recordings were found from the age of 7 days. These young mice were studied in a special apparatus in which electrocorticogram and whole body movements were recorded. This involves other criteria for seizures than those used in the investigation reported here, which may account for discrepancies.

The experimentally found higher CD50 values for pentetrazol in mg/kg in young mice compared with adults are in general agreement with the rise in values calculated theoretically according to body surface area. Thus the high CD50 values in 1-day-old and 3-day-old mice can be partly explained by the relatively large body area of these animals. However the discrepancy between the actual and the calculated values suggests that some other factor may also be of importance

Pentetrazol seems to act on cortical as well as subcortical structures (HAHN 1961) It is possible that these and other structures responsible for the spread of impulses are less developed and more difficult to activate in the newborn. In fact BERNHARD *et al* (1962) found in foetal sheep after evoking epileptic activity in the EEG by electrical cerebral stimulation or by pentetrazol injection, that no efferent impulses could be recorded from the sciatic nerve The situation in newborn mice could be similar to that in foetal sheep, especially since the sheep is born relatively mature.

Different opinions are held on the permeability of the blood-brain barrier during development (For review see DAVSON 1963) As little is known about the fate of pentetrazol in the body in adult animals, the importance for the convulsant effect of pentetrazol of absorption, penetration of the blood-brain barrier and excretion through the kidneys in growing mice is not clear An impression of the absorption factor is gained from the induction times in table 2. The absorption from subcutaneous tissue is roughly similar in 1–5 days old mice, later increasing, and most rapid at the age of 3 weeks Why this is so cannot at present be explained Penetration into the brain is included in these induction times. The mutual order of the induction times does however not bear any relation to the corresponding CD50 values these, for instance, are similar for 3 week old and adult mice, althouth the absorption rate is different.

For the barbiturates the same factors could be reckoned with as for pentetrazol A factor of special importance for such a drug is the "peak effect" i e. when it has its maximal effect To get information about this, three different intervals between the injection of phenobarbital and pentetrazol were chosen. It is evident from fig. 2 that the peak effect of phenobarbital varies with age. For 3–5 days old mice it is closer to 50 minutes, and from 9 days it is closer to 30 minutes.

But when the theoretical ED50 values according to body surface are calculated, it can be seen from fig. 2 that the experimentally found values in all three experiments are smaller than what would be expected even when the peak effect gives a low ED50 value in the adult animals. Not enough is known about the mechanism of action of phenobarbital to tell whether that could account for the age variation.

About the distribution of phenobarbital in the brain in growing animals, there is an interesting study of DOMEK et al (1960). They showed with isotope labelled phenobarbital in kittens, that the drug is primarily localized in the potential white matter of newborn animals, whereas during maturation the drug becomes more and more localized in the grey matter If this is also true for the mouse, it could perhaps be related to the greater effect of this drug against pentetrazol in the youngest mice

Mephobarbital compared with phenobarbital (injection interval 50 minutes) shows an almost identical variation of anticonvulsant effect with age which confirms some of the clinical impressions about the usefulness of this drug in childhood epilepsy of grand mal type

Conclusions from these experiments must be cautiously drawn because the variable measured was based on the observation of seizures. Subclinical seizures with cerebral epileptogenic activity may have occurred in animals not seen to have convulsions. Further studies are therefore desirable.

Summary

Age-related variations in convulsant and anticonvulsant effects of pentetrazol, phenobarbital and mephobarbital have been studied in mice during maturity The median convulsant doses of pentetrazol were determined in 1 3 5, 7 9 12, and 21 day-old and adult male mice and were found to decrease to about ½ neonatal value (in mg/kg) from birth to 9 days. After 9 days the convulsant dose was constant The median anticonvulsant doses for phenobarbital antagonizing pentetrazol-induced fits in 1 3 5 9 and 21 day-old and adult male mice were determined for three different intervals between injections. The age related variation was found to be dependent on this interval. A comparison with theoretically calculated values corresponding to body area showed that the anticonvulsant doses in newborn animals were about ½–¼ of what could be expected from body area. The median anticonvulsant doses of phenobarbital and mephobarbital against pentetrazol (in mg/kg) had an almost identical age variation during maturity

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The Occurrence of Homovanillic Acid in Human Brain

By

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(Received March 31 1965)

There is substantial support for the view that dopamine (DA) serves as a transmitter in the brain Most of the DA present in the brain seems to be *localized in neurons in the putamen* and the caudate nucleus smaller amounts, however are also found in the mesencephalon and the hypothalamus (BERTLER & ROSENGREN 1959 BERTLER 1961) DA is inactivated in the brain by monoamine oxidase (MAO) to 3 4-dihydroxyphenylacetic acid (DOPAC) and by catechol-O-methyltransferase (COMT) to 3-methoxytyramine Both these metabolites have been shown to occur in mammalian brain (ROSENGREN 1960 ANDÉN *et al* 1963b CARLSSON & WALDECK 1964) The end product when the amine has been metabolized *both by MAO and COMT is homovanillic acid (HVA) This* acid has been detected in mammalian brain (SHARMAN 1963 ANDÉN *et al* 1963c) and has also been found in the cerebrospinal fluid of man (ANDÉN *et al* 1963a)

It has earlier been shown that monoamines and their metabolites can be studied in the human brain even after death if too long a time has not elapsed between death and autopsy Because of this we decided to examine for their content of HVA human brains obtained from autopsy material

Material and Methods

Brai material from man was collected in the autopsy room. I 15 examined the time between death and utopsy had varied from 2 to 1 hours. The patients had died either from infections or from diseases of the circulatory system. In one an advanced Parkinsonism *after encephalitis* was diagnosed before death. I no others were any diseases present that could, as far as is known, nterfere with monoamine metabolism Nor had the patients received ny medical treatment known t affect the metabolism. As w had noted during preliminary tests suspiciously low alues in patients with dementia senilis, choose the material so as to include 7 patients who before death had show signs of advanced dementia senilis and of a reduction in motor functions and 7 patients who before death had shown no signs of this type.

The concentration of *HVA* different parts of the brain w *determined by the method*

of ANDÉN *et al* (1963 c) In most specimens the caudate nucleus, the putamen, the globus pallidus, the mesencephalon nd the hypothalamus were examined. In one the mesencephalon was divided into three parts the dorsal part, the nucleus ruber and the substantia nigra and th HVA concentrations of all these were determined. Other parts of the brain also (the thalamus, the motorcortex, the hippocampus, the pons, the medulla oblongata and the cerebellar cortex) were examined for their contents of HVA. Sometimes DA was determined on these specimens, when HVA was determined simultaneously

I order to establish the identity of the substance determined, the activation and the fluorescence spectra of all samples were compared with those of synthetic HVA in n Aminco Bowman spectro-photo-fluorometer Identificatio by means of high voltage paper electrophoresis was also sometimes carried out. The homogenates from the brain pieces were then divided into three parts. The HVA content of ne was determined in the ordinary way A small amount of HVA was added to one of the others In this and in the third the HVA was extracted and purified essentially by the method mentioned above, but instead of the tris-buffer 0.1 M $NaHCO_3$ was used. The HVA was extracted into this solution. The $NaHCO_3$ solution was sucked off and made acid with 0.5 ml 6N-HCl. This solution was then saturated with NaCl, and the HVA was extracted with ether The ether was evaporated to dryness, and the residue was extracted with 50 µl of pyridine buffer pH 6.0 or 3.5 for the high voltage paper electrophoresis. Two portions of 10 µl of pyridine were put on the paper together with a reference sample that contained synthetic HVA. The paper was cut into two pieces, one containing the reference solution and one part of the extracts from brain, the other containing the other part of the brain extract. The former was sprayed with diazotized p-nitraniline. The electrophoresis was done as described by STUDNITZ & HANSSON 1959 A strip at the place for the HVA spot was cut out on the other piece of the paper This strip was extracted with 0.001 N HCl, and its content of HVA were determined after oxidation by the method of ANDÉN *et al.* (1963 c)

Paper chromatography was done in system containing isopropanol, mmonia solution and water (80 18) (SHARMAN 1963) after purification carried out as for the electrophoresis, but final extraction of the residue was conducted with acetone instead of with pyridine solution.

Results

As shown in the table, the highest values of HVA were found in the neostriatum. In the putamen the mean value was 4 95 µg/g and in the caudate nucleus somewhat lower 3.31 µg/g. Considerable amounts of HVA were also found in the globus pallidus, in the mesencephalon and in the hypothalamus, the mean values being 2.57 1 18 and 0.76 µg/g respectively HVA was also found in other parts of the brain. Fairly high amounts were recorded in the thalamus (mean value 0 57 µg/g) and the medulla oblongata (mean value 0 54 µg/g). Somewhat lower values were detected in the hippocampus, the motor cortex and the pons (table 1), whereas the value in the cerebellar cortex was almost zero

The main part of the HVA in the mesencephalon seems to be located in the substantia nigra. In this nucleus, the value was 2.7 µg/g, whereas the nucleus rubor contained 0 45 µg/g and the dorsal part of the mesencephalon 0.27 µg/g in the one sample analysed.

Table 1

Concentration of HVA in different parts of human brains. Figures indicate the mean values ± S.E.M. Figures within brackets refer to the number of subjects.

	μg/g
N. Caudatus	3.31 ± 0.245 (13)
Putamen	4.95 ± 0.545 (14)
Globus pallidus	2.57 ± 0.193 (8)
Mesencephalo	1.18 ± 0.105 (10)
Hypothalamus	0.76 ± 0.015 (7)
Thalamus	0.57 ± 0.156 (8)
Medulla oblongata	0.54 ± 0.070 (7)
Hippocampus	0.23 ± 0.053 (8)
Motor cortex	0.19 ± 0.045 (5)
Pons	0.18 ± 0.045 (11)
Cerebellar cortex	0.08 ± 0.017 (4)

There seems to be a clear cut difference between the values of HVA in the neostriatum of the group with symptoms of dementia and the group without this type of symptom. The former group had low values: the mean value in the nucleus caudatus was 2.73 ± 0.229 μg/g (6 cases) and in the putamen 3.55 ± 0.265 μg/g (7 cases). The corresponding values in the group without symptoms of dementia were in the caudatus 3.80 ± 0.313 μg/g (7 cases) and in the putamen 5.15 ± 0.820 μg/g (7 cases).

In the brain from the patient with Parkinsonism the concentrations of HVA were low. In the putamen the value was 0.48 μg/g, in the nucleus caudatus 0.48 μg/g, in the globus pallidus 1.04 μg/g and in the mesencephalon 0.0 μg/g.

The DA determinations showed that DA is found, as is well known, at the highest concentrations in the neostriatum. Smaller amounts are found in the mesencephalon and in the hypothalamus. In some of our series the globus pallidus, the pons, the medulla oblongata and the hippocampus were examined for DA. No DA could be detected, however, by the method used (HÄGGENDAHL 1963).

The result of the identification tests was that the substance determined showed the same characteristics as did authentic HVA by both paper chromatography and high voltage paper electrophoresis. In all the determinations, the spectra obtained from the brain extracts were almost identical with those of authentic HVA. The fluorescence obtained on the eluates from the paper chromatography also agreed well with that of authentic HVA.

Discussion

That the highest HVA concentrations were found in the neostriatum is not surprising, as these nuclei contain DA and as it is known that HVA is the end product of DA metabolism Moreover in the mesencephalon and in the hypothalamus HVA may be considered to result from DA metabolism The finding that the mesencephalitic HVA is located in the substantia nigra also supports this assumption, because the dopaminergic nerve terminals in the neostriatum are known to originate from nerve cells in the substantia nigra (ANDÉN *et al* 1964 BERTLER *et al* 1964). The somewhat high values in other parts of the brain, especially in the globus pallidus, however are surprising, as no DA can be detected in these parts. In the globus pallidus the presence of neurons which have an intimate connection to dopaminergic nerves of the neostriatum might be the explanation. In other parts of the brain, where no DA is present HVA is perhaps formed as a byproduct of NA metabolism. One way to explain the HVA content would be to assume the occurrence of an unknown substance that also has HVA as an end metabolite

From the subject with Parkinsonism all the samples examined showed low values, and no HVA could be detected in the mesencephalon This supports the assumption that in Parkinsonism the dopaminergic systems in the mesencephalon and the neostriatum is disturbed (CARLSSON 1964).

One result of the investigation is that patients with advanced dementia senilis and with disturbances of motor functions appear to have low HVA values in the neostriatum. Further it seemed as though these low HVA values could be correlated better with the intensity of dementia symptoms and reduction of motor functions than with the age of the patients These questions are to be further investigated.

Acknowledgements

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From the central laboratory the FILADELFIA colony Dianalund,

Determination by Thin-Layer Chromatography of Phenytoin in Serum in the Presence of Barbiturates and other Antiepileptic and Various Drugs

By

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(Received March 17 1965)

Two methods are commonly used for routine determinations of phenytoin in serum, viz. that of DILL *et al* (1956) and that of SVENSMARK & KRISTENSEN (1963) the latter is based on the procedure of PLAA & HINE (1956) the direct measurement of the absorption in ultraviolet light.

The method of DILL *et al* (1956) is time-consuming, and I am unaware of any systematic investigations into its specificity SVENSMARK & KRISTENSEN (1963) studied the effect of a number of substances on their method of determination, both *in vivo* and *in vitro* They found that many drugs interfered with the determination primidone (mysoline ®), ethosuximide (zarondan ®), and according to the present author's investigations, N-(4 -sulphamylphenyl)-butansultam-(1 4) (ospolot ®) are among them, and it is these very medicaments that in recent years have been much used along with phenytoin in the treatment of epilepsy It was also found that large concentrations of sulphonamides interfered with the determination. Further experience shows that both hospitalized patients and out-patients are not always accurate in their reports of drug consumption, including that of contaminating barbiturates.

MACHATA & KISSER (1962) use infrared spectroscopy for certain identification of phenytoin, because no previously discribed detection agent was found able to distinguish between phenytoin and barbiturates this finding is in agreement with my own results

For these reasons, we have worked out a method that is not more accurate than that of SVENSMARK & KRISTENSEN (1963), but has a far greater specificity It must be considered more accurate, however in the range 1 0–10 mg/l, for with other methods serum blank values in this range can give rise to a large percentage error

The method is applicable both as a check on phenytoin concentrations

found by spectrophotometric methods, in which case only a single determination need be made, and when information about the medicine the patients are receiving has made it clear that determinations by other methods would prove impossible or inaccurate a duplicate determination is then carried out.

Method

Procedure

Shake 3 ml of serum +0.1 ml of concentrated HCl vigorously for 10 seconds with 25 ml chloroform, add pproximately 10 g anhydrous sodium sulphate, and repeat the vigorous shaking for pproximately 10 seconds. For duplicate determinations 3 ml nd 1 ml of serum are extracted. Filter the chloroform int 100 ml Erlenmeyer flasks, and evaporate to dryness n a waterbath (approximately 80°) in a stream of air Treat the residue with 10 ml of boiling 0.01 N HCl, and cool the flasks down to 4 in refrigerator Filter the aqueous extract through premoistened filters into 50 ml separatory funnels. Rinse the flasks and funnels afterwards with 3 ml cold 0.01 N HCl.

The extraction is repeated by shaking with 25 ml $CHCl_3$ for 30 seconds, and the chloroform layer is filtered i to 50 ml Erlenmeyer flasks through filters to which a small amount of dried sodium sulphate has been applied. The chloroform is evaporated in the usual manner The residue is transferred to a centrifuge tube with 2 × 2 ml $CHCl_3$ and the solutio is evaporated to dryness n a stream of air The residue is dissolved in 40 µl chloroform–methanol 90 10 /v The solution is then applied to chromatographic plates apply 3 µl of the test solution to the left half of the plate (see fig 1), to which standards containing 1 2, 3 4 nd 5 µg phenytoin have previously been applied, and apply 9 µl of the test solution t the right half to which standards containing 4, 6, 8 and 10 µg phenytoin have previously been applied

By this means, 4 samples ca be placed on each plate f the analysis is performed as a control, and two samples can be placed on each plate for a d plicat determination.

Preparation of plates 10 × 20 cm glass plates are prepared by the standard method described by STAHL (1967). Adsorption substance Kieselgel G (Merck) to which Leucht pigment ZS S per (Riedel de Haen) has been added.

Solvent mixture $CHCl_3$ 66 ml, methanol 33 ml, ammonia solution (25%) 1 ml Chamber Glass vessel (Desaga) with its inner surface covered by filter paper A glass trough of the same length the vessel is placed on the bottom of the vessel The trough is filled with ammonia solution (25%).

Equilibration time at least 2 hours.

Developi g time pproximately 10 minutes.

Length of run 6 cm.

Identification The plates are nspected in ultraviolet light and fluorescent spots, including phenytoin pots, are marked off

The plates are dried thoroughly in stream of hot air and preliminary comparison is made in U V light between standard spots and phenytoin spots from the specimens. The powder side of the glass plate is now treated with hot piperidine vapour by placing the plate on warm flatbottomed vessel contai ing piperidine for 1½ mi utes The plate is then treated with $CHCl_3$ apour in the same way until it is thoroughly moistened, which ill take approximately 30 seconds, and irradiated under ultraviolet lamps for ppro imately 15 minutes, so that some piperidine is dissociated and the plate stained yellow the contrast between fluorescent spots and background is retained.

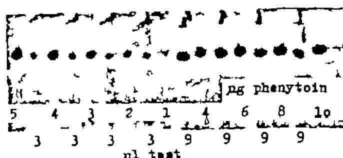


Fig. 1 Photograph in U V light of stained plate to illustrate the used system of pplication. Four different sera, added to each with 10 µg/ml phenytoin have been subjected to the standard procedure.

The plates are now moistened throughly with a 1 / aqueous $CuSO_4$ solution and left t dry at room temperature. The phenytoin spots are thereby stained yellow certal barbiturates at higher concentrations are stained blue, and the background is a faint pale green.

Results

Quantitative determination

A visual evaluation can be made with sufficient accuracy on the lines described below if necessary in ultraviolet light, when the contrast between background and spots is best.

Prilliminarily rough determination is made by means of table 1 where the relation between the number of visible spots and serum phenytoin contents is indicated.

Table 1

The number of visible, stained spots, (indicated by +) on the chromatogram, as a function of serum phenytoin in mg/l extracted from 3 ml or 1 ml f serum.

After this rough determination a comparison is made with the standard spots. For the system of application, see fig 1 and text.

mg/l in serum	Left half of plate (3 µl appl.)		Right half f plate (9 µl appl.)	
	Serum extracted		Serum extracted	
	3 ml	1 ml	3 ml	1 ml
0 0			(+)	–
2 0–5			+	(+)
6 18	+		+	+
more tha 18	+	+	+	+

On the basis of recovery tests it was stated that each µg in the standard spots from the middle to the left will as compared to 3 µl of the unknown solution correspond to 6 µg/ml in the sample 1–5 µg will thus correspond to 6–30 µg/ml in the sample, and if 1 ml of serum is extracted this will correspond to 18–90 µg/ml. To identify down to approximately 1 µg/ml, more than 9 µl of the sample may be applied If the standard spots from the middle to the right are compared with 9 µl of the sample solution from 3 ml of serum, they will correspond to contents of 2, 8 12, 16 or 20 µg/ml.

Pronounced divergences between the sizes of the fluorescent spots and the corresponding yellow spots suggest that barbiturates with nearby Rf value are present in the sample This is not important as no barbiturates stain yellow

The stability of the developed stain is such that it will keep for at least one month in daylight.

Recovery and accuracy

Recovery tests were made, partly by adding known amounts of pheny toin, from 1–50 µg/ml, to the same serum pool and partly by adding the same amount of phenytoin to different sera. The average recovery was, after correcting for demonstrated loss, 84 percent. More than 500 phenytoin analyses were carried out by SVENSMARK & KRISTENSEN's method (1963), and comparisons were made with these values (see fig. 2) Deviations of more than 2 µg/ml were rarely found, except when the patients were under treatment with substances such as those mentioned in the introduction An accuracy of 1–2 µg/ml is soon achieved

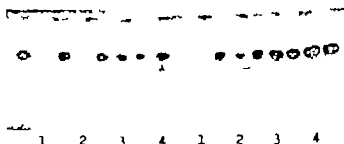


Fig Check o phenytoi concentrations found by S ENSMA nd K ISTENSEN (1963) spectrophotometric method

1 less than 2 µg/ml
2 5 9 µg/ml
3 13 µg/ml
4 .5 µg/ml

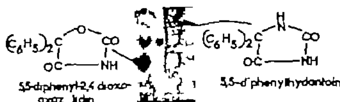


Fig. 3 Formulas illustrating the close relationship between phenytoin and epidone ® the differences Rf values are demonstrated by means of photographs in U V light of a plate before and after staining. The serum used was from patient both receiving epidone ® and phenytoin. The spot beneath that of epidone ® is metabolite of this drug, found i blood from patients under treatment.

Specificity

The antiepileptic drugs examined partly as pure substances and partly by extraction from serum from patients who had been treated with these drugs for a long period are enumerated below The WHO names are given first, with the NFN name in brackets when it differs essentially from the WHO name

Acetazolamide, barbital (diemalum), chloral hydrate, epidone ® (5,5 diphenyl-2,4 dioxo-oxazolidine), ethotoin ethosuximide, mephenytoin, metharbital (endiemalum) neo-citrullamon ® (α amino-δ-diphenyl hydantoyl valeric acid), ospolot ® (N-(4 -sulfamylphenyl)-butansultam (1 4), phenobarbital (phenemalum), phensuximide, trimethadione, and G 32883 ® (5-carbamyl-5 H-dibenzo (b,-f) azepin)

Determinations were also made on serum from patients treated with various drugs, psychopharmaca, antipyretics, sedatives (including other barbiturates), sulphonamides, etc. So far no substance has been found to give rise to confusion in, or interference with, the determination.

The only substances found to develop a similar yellow stain are 5-(p-hydroxyphenyl) 5-phenylhydantoin, shown by BUTLER (1957) and MAYNERT (1960) to be one of the most significant metabolites of phenytoin, and epidone ® But the difference between the Rf value of these substances and that of phenytoin is so pronounced that then confusion is not possible (see fig. 3)

Discussion

LOUS (1945) and BJERRE & PORTER (1963) have shown that no great yield is obtained by acidifying serum before the extraction of those barbiturates that do not differ from phenytoin in the extent of their recovery BJERRE & PORTER have further shown that a shaking time of 2×10 seconds is sufficient to give quantitative extraction, if the proteins have been precipitated with sodium sulphate. It is our experience that the

addition of HCl is advantageous when the extract is to be used for chromatography as this will decrease the amount of undesirable substances soluble in $CHCl_3$. It is necessary to purify the evaporated $CHCl_3$ extract before application to the plates, if uniform Rf values are desired for serum extracts and pure substances. Moreover fatty substances from serum will be deposited on the sides of the glass, with the result that quantitative solution of the residue from evaporation becomes impossible in so small an amount of liquid as 40 µl EBERHARDT *et al* (1962) suggested the use of active carbon and alumina for the purification In our experience the yield so obtained will be irregular and the procedure is therefore not applicable to quantitative analysis. Florisil ® has likewise been employed (PETZOLD *et al* 1963 STOKES *et al* 1962) it is then particularly the more acid components as well as some chromogens that are removed. As we do not wish to remove salicylic acid or other acid drugs, and as this purification has not always proved adequate in practice, we have employed the method sketched by MAEHLY & LINTURI (1967) for removal of fatty substances by transferring the desired substances to the aqueous phase.

Re-extraction from 0 01 N HCl to $CHCl_3$ was studied It was found that after no more than 20 manual shakings, corresponding to approximately 10 seconds, more than 99 % of phenytoin added was in the organic phase.

Complete recovery was not attempted, so as to reduce the number of operations, since the described method has to be used for routine purposes.

In agreement with PAULUS (1963), we did not find it advantageous to employ buffered plates or to employ alumina instead of Kieselgel or mixtures of these substances.

A strong eluant (33 % methanol) was preferred, thus avoiding a great number of neutral substances that show fluorescence in ultraviolet light, upsetting the inspection in ultraviolet light before staining. KELLEHER & ROLLASON (1964) employ a liquid with a methanol content of approximately 20 % for separating barbiturates. The liquid used in our study is likewise applicable for this purpose, and a modification of the procedure can be employed for determining p-hydroxyphenytoin in urine, barbiturates in mixture, as well as barbiturates in the presence of sulphonamide It is intended to discuss these procedures in a subsequent study

Summary

A visual method is described for the quantitative and qualitative determination of phenytoin in serum by means of thin layer chromatography

The staining reaction was the formation of a yellow Cu^{++} piperidine-phenytoin complex, whose specificity has been studied.

No other commonly employed anticonvulsive agents interfered including other hydantoin preparations and barbiturates.

Serum was examined from patients receiving drugs of widely differing kinds so far no substance has been found to interfere with the determination

When 3 ml serum is used, the limit for determinations is 1 mg/L The average recovery was 84 %. The accuracy of visual evaluation is 1–2 µg/ml.

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Names approved by
The Nordic Pharmacopoeia Council[1])
(NFN Names)

(Received March 27 1965)

The Nordic Pharmacopoeia Council announces its approval of NFN[2]) names for the substances in the list below These names may be used should the substances be included in the Nordic Pharmacopoeia, in one or more of the Nordic official formularies or in addenda to any of these.

The substances for which these names have been approved are identified by giving their chemical names first, secondly the most frequently used non-proprietary names, including the International Non Proprietary Names, approved by WHO and thirdly a selection of the best known registered trade names and other names of pharmaceutical specialities (preceded by the letter ®) applying either to the substance itself or to salts of it or to preparations of which it is the active principle. This information is given in a similar manner to that in the Council's publication "NFN-navne"[3]) both in volume of information and in use of abbreviations[4])

Names approved in 1964[5])

NFN-name	*Other Names*
Aceclidinum (pINN)	Acetic acid quinuclidinyl-(3)-ester (Parasympatomimetic)
Acetiaminum (pINN)	Acetic acid ester of N-((4-amino-2-methylpyrimidinyl-(5))methyl)-N-(2-acetylthio-4-hydroxy-1-methylbuten-(1)-yl)formamide. (Thiamine effect)
Acetryptinum (pINN)	2-(5-Acetylindolyl-(3))ethylamine = 5-acetyl-tryptamine. (Antihypertensive)

[1]) The General Secretary Pack, Stockholm 60, Sweden.

[2]) NFN Abbreviation of the Nordic name ("Nordiska Farmakopénämnden") for the Nordic Pharmacopoeia Council.

[3]) NFN-navne, 2nd edition, Copenhagen, Helsingfors, Reykjavik, Oslo and Stockholm, with Addendum 1961

[4]) The abbreviation USAN" indicates, that the name is approved by the American Medical Association United States Pharmacopeia Nomenclature Committee.

[5]) Names pproved in 1963 see this journal 1964 21 123–138.

NFN name	*Other Names*
Acetylcysteinum (pINN USAN)	N-Acetyl-L-cysteine. ® Mucomyst. (Mucolytic)
Acidum aminocaproicum (BAN pINN USAN)	6-Aminohexanoic acid. ® Amicar Caprocid, Eaca, Epsikapron, Epsilon-Aminocapronsyre. (Antifibrinolytic)
Acidum clofenamicum (pINN)	N-(2,3-Dichlorophenyl)anthranilic acid. (Antirheumatic)
Acidum flufenamicum (pINN USAN)	N-(3-(Trifluoromethyl)phenyl)anthranilic acid. ® Arlef. (Antirheumatic)
Acidum fusidicum (BAN pINN)	Antibiotic, produced by Fusidium coccineum. ® Fucidin, Fucidina.
Acidum fyticum (pINN USAN)	Cyclohexanehexol-(1,2,3 4,5 6)-hexaphosphoric acid ester = inositol hexaphosphoric acid ester = phytinic acid. USAN Sodium Phytate (sodium salt). ® Rencal. (Treatment of hypercalciuria)
Acidum kainicum (pINN)	-Carboxy-4-isopropenylpyrrolidine-(3)-acetic acid. (Anthelmintic)
Acidum mefenamicum (BAN pINN USAN)	N-(2,3-Xylyl)-anthranilic acid. ® Ponstan. (Antirheumatic)
Acidum metrizoicum sodium salt natrii metrizoas (BAN pINN)	3-Acetamido-2,4 6-triiodo-5-(N-methylacetamido)-benzoic acid BAN Sodium Metrizoate, pINN Natrii metrizoas (sodium salt). ® Isopaque, Triosil. (X-ray contrast medium)
Acrisorcinum (pINN)	9-Aminoacridinium-4-hexylresorcinolate = salt of aminoacridine and hexylresorcinol. ® Akrinol. (Treatment of tinea versicolor)
Alazani triclofenas (pINN)	3-Ethyl-2-(3-(3-ethylbenzothiazolinylidene-(?))propene-(1)-yl)benzothiazolium-2,4,5-trichlorophenolat compound with 2,4,5-trichlorophenol. (Anthelmintic)
Albutoinum (pINN USAN)	3-Allyl-5-isobutyl-2-thiohydantoin. ® Euprax. (Anticonvulsive)
Aloxiprinum (BAN pINN)	Polymere condensation product of aluminium oxide and acetylsalicylic acid = basic aluminium acetylsalicylate-complex. ® Palaprin, Paloxin. (Analgetic)

NFN-name	Other Names
Althiazidum (pINN)	3-((Allylthio)methyl)-6-chloro-7-sulfamoyl-3,4-dihydro-2H-1,2,4-benzothiadiazinedioxide-(1,1). USAN Althiazide. (Diuretic)
Amfepramonum (DCF,pINN)	Diethylamino-1-phenylpropanone-(1). BAN NND Diethylpropion. Ⓡ Dietopon, Dobesin, Natorexic, Regenon, Tenuate, Tylinal. (Anorexigenic)
Ampicillinum (BAN,pINN,USAN)	(( )-6-(2-Amino-2-phenylacetamido))-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (( )-1-aminobenzyl)penicillin. Ⓡ Doktacillin, Penbritin, Pentrexyl, Polycillin. (Antibiotic)
Ampyriminum (pINN)	5-Phenylpyrimido[4,5-d]pyrimidinetriamine-(2,4,7). (Diuretic)
Anazolenum trisodium salt anazolennatrium (pINN USAN)	4-((4-Anilino-5-sulfonaphthyl-(1))azo)-5-hydroxy-naphthalenedisulfonic acid-(2,7). BAN Sodium Anoxynaphthonate, pINN Anazolenum natricum, USAN Sodium Anazolene (trisodium salt). Ⓡ Coomassie blue. (Dyestuff for diagnostic purposes)
Anilamatum (pINN)	Methylcarbamic acid ester of salicylanilide. (Antirheumatic)
Argipressinum (BAN,pINN)	8-Argininevasopressin. (Antidiuretic)
Argiprestocinum (pINN)	8-Arginineoxytocin. (Antidiuretic, oxytocic)
Azotomycinum (pINN USAN)	Antibiotic, produced by Streptomyces ambofaciens. (Antineoplastic)
Batilolum (pINN)	3-Octadecyloxypropanediol-(1,2). (Treatment of radiant sickness)
Benfotiaminum (pINN)	Phosphoric acid ester of N-((4-amino-2-methylpyrimidinyl-(5))methyl)-N-(2-benzoylthio-4-hydroxy-1-methylbuten-(1)-yl)formamide. Ⓡ Betamine. (Thiamine effect)
Bentiaminum (pINN)	Benzoic acid ester of N-((4-amino-2-methylpyrimidinyl-(5))methyl)-N-(2-benzoylthio-4-hydroxy-1-methylbuten-(1)-yl)formamide. Ⓡ Neviton. (Thiamine effect)
Benzaronum (pINN)	(2-Ethylbenzofuranyl-(3))-(4-hydroxyphenyl)ketone. (Vitamin P effect, reduces the bleeding-time)
Benzbromaronum (pINN)	(3,5-Dibromo-4-hydroxyphenyl)-(2-ethylbenzofuranyl-(3))ketone. (Spasmolytic)

NFN-name	*Other Names*
Benzchinamidum	Acetic acid ester of N,N-diethyl-2-hydroxy-9 10-dimethoxy-1,3,4 6,7 11b-hexahydro-2H-benzo[a]quinolizinecarboxamide-(3). pINN Benzquinamidum (USAN). ® Quantril. (Psycho-sedative, antiemetic)
Benzilonium bromide benziloni bromidum	3-Benziloyloxy-1 1-diethylpyrrolidiniumhydroxide. BAN USAN Benzilonium Bromide, pINN Benziloni bromidum (bromide). ®: Portyn, Ulcoban. (Anticholinergic)
Benzodepum (pINN)	Di(aziridinyl-(1))phosphinylcarbamic acid benzyl ester USAN Benzodepa. ®: Dualar *(Antineoplastic)*
Betahistinum (pINN,USAN)	N Methyl-2-(pyridyl-(2))ethylamine. USAN Betahistine Hydrochloride (dichloride). ® Serc. (Diamine oxydase inhibitor)
Betainchloralum	Chloralhydrate compound with betaine. BAN USAN Chloral Betaine. pINN: Cloralum betainum. ® Beta-chlor Somilan. (Hypnotic)
Betanidinum (pINN)	1 Benzyl-2,3-dimethylguanidine. BAN Bethanidine. USAN Bethanidine Sulfate (sulfate). ® Esbatal. (Antihypertensive)
Betoxicainum	3-Amino-4-butoxybenzoic acid-(2-(-diethylamino-ethoxy)ethyl)ester pINN Betoxycainum. ® Midicaine. (Local anaesthetic)
Bisacodylum (BAN pINN NND)	4 4 -(Pyridyl-(2)-methylene)diphenol-diacetic acid ester ® Clysodrast, Dulcolax, Perilax. *(Laxative)*
Bisbentiaminum (pINN)	D benzoic acid ester of N N {dithiobis(2-(-hydroxy-ethyl)-1-methylvinylene)]-bis[N-((4-amino-2-methyl-pyrimidi yl-(5))methyl)formamide} ® Beston. (Thiamine effect)
Bolasteronum (pINN,USAN)	17β-Hydroxy-7α,17α-dimethylandrostene-(4)-one-(3) = 7α, 17-dimethyltestosterone (Anabolic, androgen)
Bromacrylidum (pINN)	N-((3-Bromopropionamido)methyl)acrylamide (Antineoplastic)
Bufenadrinum	-(1-(2-tert Butylphenyl)-1-phenylmethoxy)-N,N-dimethylethylamine. *pINN Bufenadrinum.* (Antihistaminic)

NFN-name	*Other Names*
Butaperazinum (pINN USAN)	1 [10-(3-(4-Methylpiperazinyl-(1))propyl)-phenothiazinyl-(2)]butanone-(1). ® Randolectil. (Psycho-sedative)
Butaverinum (pINN)	3-Phenyl-3-piperidinopropionic acid butyl ester DCF Butamevérine. ® Cemora. (Anticholinergic)
Buthiazidum (pINN)	6-Chloro-3-isobutyl-7-sulfamoyl-3,4-dihydro-2H 1,2,4-benzothiadiazinedioxide-(1 1). ® Eunephran. (Diuretic)
Butopiprinum (DCF,pINN)	2 Phenyl-2-piperidino acetic acid (2-but xyethyl)ester (Antitussive)
Carsalamum (BAN pINN)	3 4-Dihydro-2H 1,3-benzoxazinedione-(2,4). (Analgetic)
Clefamidum (BAN pINN)	2,2 Dichloro-N-(2-hydroxyethyl)-N-(4-(4--nitrophenoxy)benzyl)acetamide. ® Mebinol. (Amoebicide)
Clobenztropinum (pINN)	3-(1-(4-Chlorophenyl)-1-phenylmethoxy)-8--methylnortropane. (Antihistaminic)
Clodacainum (pINN)	2'-Chloro-2-(N-(2-diethylaminoethyl)ethylamino)-acetanilide. (Local anaesthetic)
Clodantoinum	5-(1 Ethylpentyl)-3-(trichloromethylthio)-imidazolidinedione-(2,4). BAN USAN: Chlordantoin. pINN Clodantoinum. ® Sporostacin. (Fungicide)
Clofenamidum (pINN)	4-Chlorobenzenedisulfonamide-(1,3). DCF Monochlorphénamide ® Aquedux, Haflutan. (Diuretic)
Clofibratum (BAN,pINN,USAN)	2-(4-Chlorophenoxy)-2-methylpropionic acid ethyl ester ® Atromid S. (Treatment of hypercholesterolemia)
Clonitratum (pINN USAN)	3-Chloropropanediol-(1,2) dinitric acid ester ® Dylate. (Vasodilator)
Clopamidum (pINN,USAN)	4-Chloro-N-(cis-2,6-dimethylpiperidino)-3-sulfamoylbenzamide. ® Brinaldix. (Diuretic)
Clorindionum (BAN,pINN)	2-(4-Chlorophenyl)indanedione-(1,3). ® Indalitan. (Anticoagulant)

NFN-name	*Other Names*
Cloxacillinum (BAN,pINN USAN)	6-(3-(2-Chlorophenyl)-5-methylisoxazolecarboxamido-(4))-3,3-dimethyl 7-oxo-4-thia 1-azabicyclo[3,2,0]-heptanecarboxylic acid-(2) = (3-(2-chlorophenyl)-5-methylisoxazolyl-(4))penicillin. USAN Sodium Cloxacillin (sodium salt). ® Ekvacillin Orbenin. (Antibiotic)
Coumetarolum	3,3'-(2-Methoxyethylidene)-bis(4-hydroxy-2H-chromenone-(2)). BAN Cumetharol. pINN Coumetarolum. ® Dicumoxane. (Anticoagulant)
Cyamemazinum (pINN)	10-(3-Dimethylamino-2-methylpropyl)phenothiazine-carbonitrile-(2) DCF Cyamépromazine. (Psycho-sedative)
Cyclarbamatum (BAN,DCF pINN)	Cyclopentylidenedimethanol dicarbanilic acid ester ® Casmalon. (Anticonvulsive)
Cycloguanili embonas (BAN,pINN)	1-(4-Chlorophenyl)-2,2-dimethyl-1,2-dihydro-1,3,5-triazinediamine-(4,6) compound (2:1) with embonic acid USAN Cycloguanil Pamoate. (Chemotherapeutic)
Cyclomenolum (DCF,pINN)	-Cyclohexyl 3,5-xylenol (Antibacterial)
Cyclovalonum (pINN)	2,6-Divanillylidenecyclohexanone. ® Cyvalon. (Choleretic)
Desipraminum (BAN pINN USAN)	5-(3-Methylaminopropyl)-10,11-dihydro-5H-dibenz[b,f]azepine. USAN Desipramine Hydrochloride (chloride). ® Norpramin, Pertofran (Psycho-analeptic)
Deslanosidum (BAN,pINN USP)	(+)-3β-(0-β-D-glucopyranosyl-(1 → 4)-0-β-D-digitoxopyranosyl-(1 → 4)-0-β-D-digitoxopyranosyl-(1 → 4)-β-D-digitoxopyranosyloxy)-12β,14-dihydroxy-5β-cardene-(20(22))-olide = desacetyllanatoside C ® Cedilanid, Lanatosid C (Digitalis effect)
Dexoxadrolum (pINN USAN)	(+)-4-(2,2-Diphenyl-1,3-dioxolanyl-(4))piperidine USAN Dexoxadrol Hydrochloride (chloride). (Psycho-analeptic)
Diclofenamidum (pINN)	4,5-Dichlorobenzenedisulfonamide-(1,3). BAN Dichlorphenamide (DCF). (Carboanhydrase-inhibitor)
Dietroxium (pINN)	5,5-Diethyl-3,4,5,6-tetrahydro-2H-1,3-oxazinedione-(2,4). BAN Diethadione. ® Ledosten, Toce. (Psycho-analeptic)

NFN-name	*Other Names*
Difenidolum (pINN USAN)	1 1 Diphenyl-4-piperidinobutanol. (Antihistaminic, antiemetic)
Dimefadanum (pINN USAN)	N,N Dimethyl-3-phenylindanamine-(1). (Analgetic)
Dimethindenum hydrogen maleate dimethindeni maleas (pINN)	N N Dimethyl-2-(3-(1 pyridyl-(2)-ethyl)indenyl -(2))ethylamine. BAN: Dimethindene. USAN Dimethindene Maleate (hydrogen maleate). ® Fenistil, Fenostil, Forhistal Male- te. (Antihistaminic)
Dipyridamolum (BAN pINN,USAN)	2,2',2'',2'''-((4,8-Dipiperidinopyrimido[5,4- -d]pyrimidinediyl-(2,6))diimino)tetrakisethanol. ® Persantin. (Coronary vasodilator)
Disopyramidum (pINN)	2-(2 Diisopropylaminoethyl)-2-phenyl-2 -(pyridyl-(2))acetamide. (Anticholinergic)
Doxapramum (BAN,pINN,USAN)	1 Ethyl-4-(2-morpholinoethyl)-3,3-diphenyl- pyrrolidinone-(2). USAN Doxapram Hydrochloride (chloride). ® Stimulexin. (Analeptic)
Drostanololum (pINN)	17β-Hydroxy-2α-methyl-5α-androstanone-(3). USAN Dromostanolone Propionate (propionic acid ester). ® Drolban. (Androgen)
Duazomycinum (pINN USAN)	Antibiotic, produced by Streptomyces ambo- faciens = diazomycin A = duazomycin A. (Antineoplastic)
Efloxatum (pINN)	(4-Oxo-2-phenyl-4H-chromenyl-(7)-oxy)acetic acid ethyl ester ® Recordil. (Coronary vasodilator)
Epitizidum (pINN)	6-Chloro-3-((2,2,2-trifluoroethyl)thio- methyl)-7-sulfamoyl-3,4-dihydro-2H 1,2,4-benzothia- diazine-1,1-dioxide. BAN USAN Epithiazide. (Diuretic)
Ethylestrenolum (BAN)	17α-Ethylestren-(4)-ol-(17β). pINN Ethylestrenolum (DCF USAN). ® Duraboral, Orabolin, Orgabolin. (Anabolic, androgen)
Etoxazenum (pINN)	4-((4-Ethoxyphenyl)azo)phenylene-(1,3)-diamine. USAN Ethoxazene Hydrochloride (chloride). ® Carmurit, Cystural, Serenium. (Urinary antiseptic and analgetic)
Etymemazinum (DCF pINN)	10-(3-Dimethylamino-2-methylpropyl)-2-ethylphenothia- zine. ® Sergetyl. (Psycho-sedative)

NFN-name	*Other Names*
Etynodiolum diacetic acid ester etynodioli acetas (DCF pINN)	17α Ethinylestren-(4)-diol-(3β,17β). BAN: Ethynodiol. USAN Ethynodiol Diacetate (diacetic acid ester). (Gestagen)
Etyprenalinum	1-(3 4-Dihydroxyphenyl)-2-isopropylaminobutanol. BAN USAN Isoetharine. pINN Isoetarinum. ® Dilabron. (Sympatomimetic, bronchodilator)
Felypressinum (pINN)	8-Lysine-2-(phenylalanine)vasopressin. ®: Octapressin. (Vasopressin effect)
Fenbenicillinum (pINN)	3,3-Dimethyl-7-oxo-6-(2-phenoxy 2-phenyl-acetamido)-4-thia-1-azabicyclo[3,2,0] heptanecarboxylic acid-(2) = (1-phenoxybenzyl)penicillin. BAN Phenbenicillin. ® Penspek. (Antibiotic)
Fencarbamidum (pINN)	Diphenylthiocarbamic acid (S-(2-diethylaminoethyl))-ester USAN Phencarbamide. ® Escorpal. (Spasmolytic)
Fenyripolum (pINN USAN)	1-Phenyl-2-(pyrimidinyl-(2)-amino)ethanol. USAN Fenyripol Hydrochloride (chloride). (Muscle relaxant)
Fluanisonum (DCF pINN)	1-(4-Fluorophenyl)-4-(4-(2-methoxyphenyl)-piperazinyl-(1))-butanone-(1). ® Sedalande. (Psycho-sedative)
Flumetasonum (pINN)	6α,9-Difluoro-11β,17α,21-trihydroxy-16α-methylpregnadiene-(1 4)-dione-(3,20) = 6α,9-difluoro-16α-methylprednisolone. BAN USAN Flumethasone. ® Locorten. (Glucocorticoid)
Fluperolonum 21-acetic acid fluperoloni acetas (BAN,pINN USAN)	9-Fluoro-11β,17α,21-trihydroxy-21-methyl-pregnadiene-(1,4)-dione-(3,20) = 9-fluoro-21-methylprednisolone. USAN Fluperolone Acetat (21 acetic acid ester) ® Alacortril, Methral. (Glucocorticoid)
Fluprednisolonum (pINN USAN)	6α-Fluoro-11β,17α,21-trihydroxypregnadiene-(1 4)-dione-(3,20) = 6α-fluoroprednisolone. ® Alphadrol. (Glucocorticoid)
Fluracilum	5-Fluoro-1 2,3,4-tetrahydropyrimidinedione-(2,4) = 5-fluorouracile. pINN Fluorouracilum (BAN USAN). (Antineoplastic)

NFN-name	*Other Names*
Flurotylum (pINN USAN)	Bis(2,2,2-trifluoroethyl)ether ®: Indoklon. (Shock-inducing agent)
Galantaminum (DCF pINN)	9-Methoxy-2-methyl-1,2,3,4 6,7 7a,11c-octahydro- benzofuro[4,3,2-efg][2]benzazocinol-(6). (Anticholinesterase effect)
Glucaloxum (pINN)	Polymere complex of glycerol with aluminium hydroxide. BAN Glycalox. ® Manalox AG (Antacid)
Glypinamidum (pINN)	1-(4-Chlorophenylsulfonyl)-3-(hexahydro- azepinyl-(1))carbamide. (Oral antidiabetic)
Hexadilinum (pINN)	2-(2,2 Dicyclohexylvinyl)piperidine (Coronary vasodilator)
Hexamidinum (DCF,pINN)	4 4 -(Hexamethylenedioxy)dibenzamidine. ® Desomidine. (Antibacterial, local sclerosing)
Hexopyrronium bromide hexopyrronii bromidum	3-(2-Cyclohexyl-2 phenylglycoloyloxy)-1 1 -dimethylpyrrolidiniumhydroxide. pINN Hexopyrronii bromidum (bromide). (Anticholinergic)
Indometacinum (pINN)	2-(1-(4-Chlorobenzoyl)-5-methoxy-2-methyl- indolyl-(3))acetic acid. BAN USAN Indomethacin. ® Indocid, Indocin. (Antirheumatic)
Iproclozidum (BAN DCF,pINN)	2-Isopropyl-(4-chlorophenoxy)acetohydrazide. ® Sursum. (Monoamine oxydase inhibitor)
Itramini tosylas (BAN,pINN)	4-Methylbenzenesulfonate of nitric acid (2-aminoethyl)ester. ® Nilatil. (Vasodilator nitrite effect)
Iotalaminum sodium salt Iodtalaminnatrium	5-Acetamido-2,4,6-triiodo-N-methylisophthal- amidic acid. pINN Acidum iotalamicum. USAN Iothalamic Acid. ® Angio-conray Conray (X-ray contrast medium)
Leiopyrrolum (DCF,pINN)	N,N Diethyl-2-(2-(2-methyl-5-phenylpyrrolyl- -(1))phenoxy)ethylamine. ® Leiopiegil. (Anticonvulsive)
Levoglutamidum (DCF pINN)	Glutamine.
Levoxadrolum (pINN USAN)	( )-2-(2,2 Diphenyl-1,3-dioxolanyl-(4))piperidine. USAN Levoxadrol Hydrochloride (chloride). (Psycho-analeptic)

NFN-name	*Other Names*
Lincomycinum (pINN USAN)	Antibiotic, produced by Streptomyces lincolnensis. ® Lincocin.
Lynestrenolum (DCF,pINN USAN)	17α-Ethinylestren-(4)-ol-(17β). BAN Lynoestrenol. ® Orgametil, Orgametril. (Oral contraceptive)
Lypressinum (BAN pINN)	8-Lysinevasopressin. (Vasopressin effect)
Mecloxaminum (pINN)	2-(1-(4-Chlorophenyl)-1-phenylethoxy)-N,N--dimethylpropylamine. ® Isopropyl-systral. (Anticholinergic)
Mecysteinum (pINN)	Cysteine-methylester BAN Methyl Cysteine. ® Acdrile. (Treatment of chronic bronchitis)
Megestrolum (BAN pINN USAN)	17α Hydroxy-6-methylpregnadiene-(4,6)-dione-(3,20). USAN Megestrol Acetate (acetic acid ester). (Gestagen)
Melengestrolum (BAN pINN USAN)	17α-Hydroxy-6-methyl-16-methylenepregnadiene--(4 6)-dione-(3,20). USAN Melengestrol Acetat (acetic acid ester). (Gestagen)
Menotropinum	Human menopausal gonadotrophin. pINN Menotrophinum
Meraleinum sodium salt meraleinnatrium (pINN USAN)	2-(6-Hydroxy 5-(hydroxymercuri)-4,7-diiodo-3-oxo-3H xanthenyl-(9))benzenesulfonic acid. pINN Meraleinum natricum, USAN Sodium Merallein (sodium salt). ® Mercurex, Merodicein. (Antibacterial)
Meturedepum (pINN)	(Bis(2,2-dimethylaziridinyl-(1))phosphinyl)-carbamic acid ethyl ester USAN Meturedepa. ® Turloc (Antineoplastic)
Metyraponum (BAN pINN USAN)	Methyl 1,2-di(pyridyl-(3))propanone-(1). ® Metopiron, Metopirone. (Anti-aldosterone)
Molinazonum (pINN USAN)	3-Morpholino-3,4-dihydro-1,2,3-benzotriazinone-(4) (Analgetic)
Morinamidum (pINN)	N-(Morpholinomethyl)pyrazinecarboxamide-(2). ® Diazolina, Piafolina, Piazol, Piazolina. (Chemotherapeutic against tuberculose)
Nafcillinum (pINN USAN)	6-(2 Ethoxynaphthamido-(1))-3,3-dimethyl-7-oxo-4-thia 1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (2-ethoxynaphthyl-(1))penicillin. USAN Sodium Nafcillin (sodium salt). ® Unipen (Antibiotic)

NFN-name	*Other Names*
Nalidixinum sodium salt nalidixanatrium	*1-Ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-* *-naphthyridinecarboxylic acid-(3).* BAN, USAN Nalidixic Acid pINN Acidum nalidixicum. Ⓣ NeGram, Negram. *(Antibacterial)*
Naloxonum (pINN,USAN)	17 Allyl-3 14-dihydroxy-4,5-epoxymorphinanone-(6) USAN N loxone Hydrochloride (chloride) (Antimorphine effect)
Nanofinum	2,6-Dimethylpiperidine = 2,6-lupetidine. pINN Nanofinum. (Ganglion blocking)
Natrii bithionolas (USAN)	Dinatrium-2,2'-thiobis(4,6-dichlorophenolate). pINN Natrii bitionolas. USAN: Sodium Bithionolate. Ⓣ Vancide BN (Antibacterial, fungicide)
Natrii etasulfas (pINN)	N trium-2-ethylhexylsulfate. USAN Sodium Etharulfate. (Detergent)
Natrii radio-jodidum(^{131}J)	Natriumradio-iodide(^{131}J) Ph.Nord. Solutio natrii radiojodidi(^{131}J) (solution) BP Sodium Iodide (^{131}I) Solution, USP Sodium Radio-iodide Solutio (solution) (Radioactive substance)
Niclosamidum (BAN,pINN)	2',5-Dichloro-4'-nitrosalicylanilide Ⓣ Yomesan. (Anthelmintic)
Nifuralazinum	6-(5-Nitrofurfurylidenemethyl)-1,2,4-triazineamine-(3) pINN Furalazinum (Antibacterial)
Nifurazolidonum	3-(5-Nitrofurfurylideneamino)oxazolidinone-(2). pINN Furazolidonum (BAN NF). Ⓣ Furoxon, Furoxone, Tricofuron. (Antibacterial)
Nonapyrimidinum (pINN)	N-Nonyl-7H-pyrrolo[2,3-d]pyrimidineamine-(4) (Anticonvulsive)
Norethynodrelum (pINN)	17α-Ethynyl-17β-hydroxyestrene-(5(10))-one-(3) BAN USAN Norethynodrel (DCF). (Gestagen)
Octotiaminum (pINN)	Methylester of 6-acetylthio-8-[[2-[N-[((4-amino-2 methyl)pyrimidinyl-(5))methyl]formamido]-1-(2 -hydroxyethyl)propen-(1)-yl]dithio]octanoic acid. Ⓣ Neuvitan. (Thiamine effect)
Ortetaminum (pINN)	1 Methyl- -(2-tolyl)ethylamine. (Anorexigenic)
Oxadrolum	2-(2,2 Diphenyl-1,3-dioxolanyl-(4))piperidine. pINN Dioxadrolum USAN Dioxadrol Hydro- chloride (chloride). (Psycho-analeptic)

NFN-name	*Other Names*
Lincomycinum (pINN USAN)	Antibiotic, produced by Streptomyces lincolnensis. ® Lincocin.
Lynestrenolum (DCF pINN USAN)	17α Ethinylestren-(4)-ol-(17β). BAN Lynoestrenol. ® Orgametil, Orgametril. (Oral contraceptive)
Lypressinum (BAN pINN)	8-Lysinevasopressin. (Vasopressin effect)
Mecloxaminum (pINN)	2-(1-(4-Chlorophenyl)-1-phenylethoxy)-N N -dimethylpropylamine. ® Isopropyl-systral. (Anticholinergic)
Mecysteinum (pINN)	Cysteine-methylester BAN Methyl Cysteine. ® Acdrile. (Treatment of chronic bronchitis)
Megestrolum (BAN pINN USAN)	17α-Hydroxy-6-methylpregnadiene-(4,6)-dione-(3,20). USAN Megestrol Acetat (acetic acid ester). (Gestagen)
Melengestrolum (BAN,pINN USAN)	17α-Hydroxy-6-methyl-16-methylenepregnadiene- -(4,6)-dione-(3,20) USAN Melengestrol Acetat (acetic acid ester). (Gestagen)
Menotropinum	Human menopausal gonadotrophin. pINN Menotrophinum.
Meraleinum sodium salt meraleinnatrium (pINN USAN)	2-(6-Hydroxy-5-(hydroxymercuri)-4,7-diiodo- 3-oxo-3H xanthenyl-(9))benzenesulfonic acid. pINN Meraleinum natricum, USAN Sodium Meralein (sodium salt). ® Mercurex, Merodicein. (Antibacterial)
Meturedepum (pINN)	(Bis(2,2-dimethylaziridinyl-(1))phosphinyl)- carbamic acid ethyl ester USAN Meturedepa. ® Turloc (Antineoplastic)
Metyraponum (BAN,pINN USAN)	Methyl-1,2-di(pyridyl-(3))propanone-(1). ® Metopiron, Metopirone. (Anti-aldosterone)
Molinazonum (pINN USAN)	3-Morpholino-3,4-dihydro-1,2,3-benzotriazinone-(4). (Analgetic)
Morinamidum (pINN)	N-(Morpholinomethyl)pyrazinecarboxamide-(2). ® Dazolina, Piafolina, Piazol, Piazolina. (Chemotherapeutic against tuberculose)
Nafcillinum (pINN USAN)	6-(2-Ethoxynaphthamido-(1))-3,3-dimethyl-7 -oxo-4-thia-1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) = (2-ethoxynaphthyl-(1))penicillin. USAN Sodium Nafcillin (sodium salt) ® Unipen. (Antibiotic)

NFN-name	*Other Names*
Pentabamatum (pINN USAN)	3-Methylpentanediol-(2,4) dicarbamic acid ester (Psycho-sedative)
Pentalamidum (BAN,pINN)	2 Pentyloxybenzamide. (Fungicide)
Periciazinum (pINN)	10-(3-(4-Hydroxypiperidino)propyl)phenothiazine-carbonitrile-(2) BAN Pericyazine. DCF Propériciazine. Ⓣ Neulactil. (Psycho-sedative)
Picloxydinum (BAN,pINN)	1 1 -(Piperazinediyl-(1 4)-dicarbonimidoyl)-bis(3-(4-chlorophenyl)guanidine). (Antibacterial)
Pimetinum (pINN,USAN)	2-(4-Benzylpiperidino)-N N-dimethylethylamine. USAN Pimetine Hydrochloride (chloride). (Cardio-vascular agent)
Poldini methylsulfas	2 Benzilloyloxymethyl-1 1-dimethylpyrrolidinium methylsulfate. BP USAN Poldine Methylsulfate. pINN Poldini methylsulfas. Ⓣ Nactate, Nacton. (Anticholinergic)
Prednisolamatum (BAN,pINN)	21-(2 Diethylamino acetic acid)ester of 11β,17α,21-trihydroxypregnadiene-(1 4)-dione-(3,20) — 21-(2-diethylamino acetic acid) ester of prednisolone. Ⓣ: Deltacortril DA. (Glucocorticoid)
Prednylidenum (pINN)	11β,17α,21 Trihydroxy-16-methylenepregnadiene-(1,4)-dione-(3,20). Ⓣ Decortilen. (Glucocorticoid)
Propetandrolum (DCF pINN)	3-Propionic acid ester of 17α-ethylestrene-(4)-diol-(3β,17β). Ⓣ Solevar (Anabolic)
Propicillinum (BAN,pINN)	3,3-Dimethyl-7-oxo-6-(2-phenoxybutyramido)-4-thia-1-azabicyclo[3,2,0]heptanecarboxylic acid-(2) — (1 phenoxypropyl)penicillin. Ⓣ Brocillin, Celtacillin, Cetacillin, Ultrapen. (Antibiotic)
Prosultiaminum (pINN)	N-((4-Amino-2-methylpyrimidinyl-(5))methyl)-N-(4-hydroxy-1-methyl-2-(propyldithio)-buten-(1)-yl)formamide. DCF Prosulthiamine. Ⓣ Alinamin, Nevriton. (Thiamine effect)
Pyrrocainum (pINN,USAN)	2',6' Dimethyl-2-pyrrolidinyl-(1)-acetanilide. Ⓣ Endocaine. (Local anaesthetic)
Quingestronum (pINN USAN)	3-(Cyclopentyloxy)pregnadiene-(3,5)-one-(20). (Gestagen)

NFN-name	*Other Names*
Radio-aurum(^{198}*A*)	Radioactiv ^{198}Au. Radio-gold(^{198}Au).
	pINN Radio-aurum (^{198}Au)-colloidale (colloidal form).
	USP Radiogold Solution (solution).
	® Aureoloid-198
	(Radioactive substance)
Radio-cycobaminum(^{60}*Co*)	( )-α-(5,6-Dimethylbenzimidazolyl)cobamide-^{60}Co-cyanid
	pINN Radiocyanocobalaminum (^{60}C).
	(For diagnostic purposes)
Radio-tolpovidonum(^{131} *J*)	α-(4-Iodo-^{131}I-benzyl)-derivate of polyvinylpyrrolidone = α-(4-iodo-^{131}I benzyl)-2-(?-oxopyrrolid nyl-(1))-ethamer
	pINN Radiotolpovidonum. USAN Tolpovidone I^{131}
	® Raovin.
	(Radioactive substance for differential diagnosis of source of hypoalbuminaemia)
Renytolinum (pINN)	4-(Fluorenylidene-(9)-methyl)benzamidine.
	USAN Paranyline Hydrochloride (chloride).
	(A tirheumatic)
Rif mycinum (BAN,pINN)	Antibiotic, produced by Streptomyces mediterranei.
	® Rifocyn.
Sparsomycinum (pINN USAN)	Antibiotic, produced by Streptomyces sparsogenes.
	(Antineoplastic)
Sparteinum (pINN USAN)	( )-Dodecahydro-7 14-methano-2H,6H-dipyrido-[1,2-a 1 ,2′-e][1,5]diazocine.
	USAN Sparteine Sulf te (sulfat)
	® *Tocosamine Sulfate.*
	(Uterus-stimulating)
Spectinomycinum (BAN pINN USAN)	Antibiotic, produced by Streptomyces spectabilis.
	® Trobicin.
Stilbazium iodid stilbazi jodidum	1 Ethyl 2,6-bis(4-pyrrolidinyl-(1)-styryl)-pyridiniumhydroxide.
	pINN Stilbazii iodidum, USAN Stilbaz m Iodide (iodide).
	® Monopar
	(Anthelmintic)
Streptoniazidum (DCF pINN)	(*all-trans-2,4-D guanidino-3 5 6-trihydroxy-*cyclohexyl)-5-deoxy-2-0-(2-deo y -methylamino-α-L glucopyranosyl)-β- -lyxo-pentofuranosidcarbaldehyde -(3)-isonicotinoylhydrazone = isonicotinoylhydrazone of streptomycinum
	BAN, USAN Streptonicozid.
	® Streptohydrazid
	(Chemotherapeutic against t berculosis)
Sucralox um (BAN pINN)	C mplex of sucrose with aluminium hydroxide
	® M nalo AS.
	(Antacid)

NFN-name	*Other Names*
Sultiamum (pINN)	2-(4-Sulfamoylphenyl)-tetrahydro-1,2-thiazinedioxide-(1,1). BAN USAN Sulthiame. ® Contravul, Ospolot, Trolone. (Anticonvulsive)
Teclozanum (pINN USAN)	N,N'-(Phenylene-(1,4)-dimethylene)bis(2,2-dichloro-N-(2-ethoxyethyl)acetamide). ® Falmonox. (Amoebicide)
Tiabendazolum (pINN)	2-(Thiazolyl-(4))benzimidazole. USAN Thiabendazole. ® Thibenzole. (Anthelmintic)
Tiemonium iodide: tiemonii jodidum	4-(3-Hydroxy-3-phenyl-3-(thienyl-(2))propyl)-4-methylmorpholiniumhydroxide. DCF Tiemonium. pINN Tiemonii iodidum (iodide). ® Visceralgine. (Anticholinergic)
Tifenamilum (pINN)	Diphenylthioacetic acid S-(2-diethylaminoethyl)ester. USAN Thiphenamil Hydrochloride (chloride). ® Trocinate. (Anticholinergic)
Timerfonatum sodium salt: timerfonatnatrium	4-(Ethylmercurithio)benzenesulfonic acid. pINN Natrii timerfonas, USAN Sodium Thimerfonat (sodium salt). ® Sulfo-merthiolate. (Antibacterial, local treatment)
Tolboxanum (pINN)	5-Methyl-5-propyl-2-(4-tolyl)-1,3,2-dioxaborinane. ® Clarmil. (Psycho-sedative)
Tolpropaminum (BAN,pINN)	N,N-Dimethyl-3-phenyl-3-(4-tolyl)propylamine. ® Tylagel. (Antihistaminic)
Tolpyrramidum (pINN)	N-Tosylpyrrolidinecarboxamide-(1). (Oral antidiabetic)
Triclofosum (BAN,pINN,USAN)	(2,2,2-Trichloroethyl)dihydrogen phosphate. USAN Sodium Triclofos (monosodium salt). ® Tricloryl. (Hypnotic)
Trimetamidum (pINN)	N-(2-Amino-6-methylpyridyl-(3)-methyl)-3,4,5-trimethoxybenzamide. (Hypotensive)
Trimipraminum (BAN,pINN)	3-(10,11-Dihydro-5H-dibenz[b,f]azepinyl-(5))-N,N,2-trimethylpropylamine. DCF Triméprimine. ® Surmontil. (Psycho-analeptic)

NFN-name	*Other Names*
Trometamolum (pINN)	2-Amino-2-(hydroxymethyl)propanediol-(1,3). USAN Tromethamine. ® Talatrol. (Treatment of acidemia)
Troxonium tosylate troxonii tosylas	N,N,N-Triethyl-2-(3,4,5-trimethoxybenzoyloxy)ethylammoniumhydroxide. BAN Troxonium Tosylate, pINN: Troxonii tosylas (tosylate). (Antihypertensive)
Troxypyrrolium tosylate troxypyrrolii tosylas	1 Ethyl-1-(2-(3,4,5-trimethoxybenzoyloxy)ethyl)-pyrrolidiniumhydroxide. BAN Troxypyrrolium Tosylate, pINN Troxypyrrolii tosylas (tosylate). (Antihypertensive)
Tyloxapolum (BAN, pINN USAN)	Polymere product of 4-(1,1,3,3-tetramethylbutyl)phenol, ethylene glycol and formaldehyde. ® Alevaire, Superinone. (Detergent)
Uramustinum (BAN pINN)	5-Bis(2-chloroethyl)amino-1,2,3,4-tetrahydropyrimidinedione-(2,4). USAN Uracil Mustard. (Antineoplastic)
Uredepum (pINN)	Di(aziridinyl-(1))phosphinylcarbamic acid ethyl ester USAN: Uredepa. ® Avinar (Antineoplastic)
Vincristinum (BAN pINN USAN)	Alcaloid, isolated from Vinca rosea L. ® Oncovin. (Antineoplastic)
Vinleurosinum (pINN USAN)	Alcaloid isolated from Vinca rosea L. (Antineoplastic)
Vinrosidinum (pINN, USAN)	Alcaloid, isolated from Vinca rosea L. (Antineoplastic)
Virginiamycinum (DCF pINN)	Antibiotic, produced by Streptomyces virginiae. ® Staphylomycin.

Alteration in declaration

Ricinomacrogolum	The declaration is changed to Polyoxyethylene ether of castor oil.

Alterations in names

NFN name	*Previous NFN-name*
Aminophenazonum	Amidopyrinum
Choriongonadotropinum	Chorion-gonadotropinum
Natrii radio-chromas (^{51}Cr)	Natrii radiochromas (^{51}Cr)
Natrii radio-phosphas (^{32}P)	Natrii radiophosphas (^{32}P)
Serumgonadotropinum	Serum-gonadotropinum

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Distribution in Mice of ^{14}C γ-(4-methylpiperidino)-p-fluorobutyrophenone hydrochloride (FG5111), a Butyrophenone Derivative with Tranquillizing Properties

By

Niels Ebner-Jensen and Eskil Hansson

(Received March 31 1965)

It has been shown that γ-aminobutyric acid has an inhibitory effect on the central nervous system (CNS) A symposium was devoted to the subject (ROBERTS 1960) The importance of γ aminobutyric acid in the function of the CNS has not yet been fully clarified, but many attempts have been made to affect the CNS by compounds chemically similar to this substance.

From a series of syntheses based on this idea, FG5111 (γ-(4-methylpiperidino)-p-fluorobutyrophenone hydrochloride)*) was selected for further investigation after having shown tranquillizing properties similar to those of chlorpromazine and haloperidole in animal experiments (CHRISTENSEN HERNESTAM, LASSEN & STERNER 1965)

Tranquillizing effects were also established in human clinical trials (KRARUP 1965 SCHULSINGER, KIRKEGAARD NIELSEN PAULSEN, VANGTORP & GJEDDEBAECK 1965)

The investigation described below was carried out in order to study the distribution in the body and the excretion of the substance or its metabolites by the organism, especially its ability to pass the blood brain barrier

Methods

The labelled substance was synthesised by the scheme shown below

The purity of the substance was tested by paper chromatography in several systems and was at least 98 % (LARSSON, personal communication, 1965) The specific activity was 15 3 µC/mg.

*) This and the ^{14}C-labelled substance were synthesised by Ferrosan Ltd Malmö Sweden.

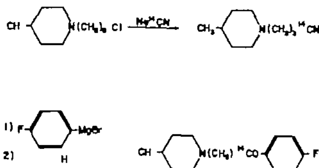


Fig. 1 Scheme of synthesis of ^{14}C FG5111

Each animal was injected intravenously with 7.5 µC (corresponding to 0.5 mg), and the radioactive compound was dissolved in 0.2 ml saline. The autoradiographic technique described by ULLBERG (1954) was used. Seven male mice (body weight 20 g) and 2 late pregnant female mice (35 g) were included in the experiment. The animals were killed by immersion in a freezing mixture of solid carbon dioxide and hexane. The interval between injection and slaughter for the male animals was 5 and 20 minutes and 1 4 48, and 96 hours. The female animals were killed 1 and 4 hours after the injection.

The frozen mice were transferred to a cold room at a temperature of −10° and sagittal sections of 20 µ and 100 µ were taken at various levels through the whole animal. Contact autoradiograms were then made by pressing the freeze-dried sections against Structurix (Gevaert)X-ray film. The time of exposure varied from 12 to 60 days.

After the development of the films, the reading of the results was made as a subjective evaluation of the blackening under the stereo-microscope. For comparison a scale of various doses of radioactivity was used (BERLIN & ULLBERG 1963)

Results

The results of the autoradiographic investigation are shown in figures 2–7 The radioactivity had largely disappeared from the blood within 5 minutes. In the autoradiograms taken at 1 and 4 hours the blood radioactivity tended to increase

Within 5 minutes the activity in the CNS had reached a high level High uptake of radioactivity were also seen in the pituitary the adrenals, the salivary glands, the lung, the kidney and the intestinal mucosa.

At 1 hour and later the radioactivity was concentrated in excretory organs such as the liver the gastrointestinal tract, the kidney and the bladder

Central nervous system (CNS)

The uptake in the CNS was high 5 and 20 minutes after the injection. The cerebral cortex, the hippocampus and the thalamus showed the highest concentration (fig. 2, 3 & 4). A decrease in radioactivity had occurred

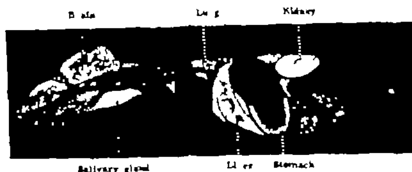


Fig. 2. Autoradiogr m showing distribution of radioactivity (light areas) 5 minutes after injection of ^{14}C FG5111 int male mouse. Note the high concentrations i brain, salivary glands, kidney liver and stomach mucosa and pituitary Excretio into the stomach via the stomach mucosa is seen The highest concentration in the CNS is seen in the grey matter



Fig. 3. Autoradiogram showing distribution of radioactivity (light areas) 20 minutes after i. v injectio of ^{14}C FG5111 int male mouse. Note the high concentration of radioactivity in the liver kidney lung nd salivary gla d. The radioactivity in the bra has decreased considerably but the cerebral cortex, the thalamus and the hippocampus retain radioactivity to a high degree.

after 1 hour and after 4 hours the radioactivity in the brain was very low The hippocampus maintained the highest concentration of the brain areas for the longest time. The concentration in the spinal cord was high at 5 and 20 minutes after the injection

Endocrine organs

A high concentration of radioactivity was seen in the pituitary and in the adrenal medulla. An especially high concentration was observed in a zone between the cortex and the medulla. Some uptake was also seen in the thyroid.



Fig. 4 Part f whole body utoradiogram showing distribution of radioactivity in the brain 20 mi utes after L injecti of ^{14}C FG5111 The highest concentration of radioactivity is seen the cerebral cortex, thalamus nd hippocampus.



Fig. 5 Autoradiogram showi g distribution of radioactivity (light reas) in a male mouse, 4 hours after injection of ^{14}C FG5111 Most of the radioactivity is seen the liver the bile d the gastrointestinal tract, indicating the marked hepatic excretion of th substance or its metabolites or both.

Respiratory organs

A high uptake was found in the lungs at 5 and 20 minutes and 1 hour after the injection High activity was also seen in the nasal mucosa

Circulatory organs

A moderate uptake was seen in the heart and the walls of the blood vessel

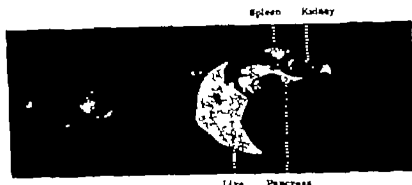


Fig 6. Autoradiogram showing distribution of radioactivity (light areas) 24 hours after i. injection of ¹ C FG5111 Some radioactivity is seen i liver pancreas, spleen, intestines and kidney Only traces are seen in other organs except the Harder's gland. In the liver and spleen high radioactivity is found in some small patches.

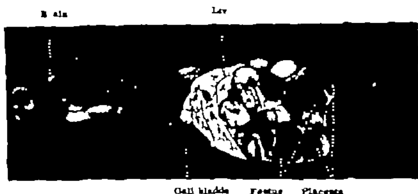


Fig 7 Autoradiogram showing distribution of radioactivity (light areas) in late pregnant mouse 1 hour after i.v injection of ^{14}C FG5111 The substance passes the placenta barrier but the concentration is lower in the foetus than i the mother Note the high concentration the gall bladder of the mother

Gastrointestinal tract

The liver accumulated a high amount of the injected compound. The highest concentration was seen from 5 minutes to 4 hours after the injec tion. Some radioactivity remained in the liver at 1 2 and 4 days after the injection and was then mainly confined to small areas (fig. 6)

A high concentration was seen in the gall bladder from 20 minutes to 4 hours after the injection (fig. 5). The gastrointestinal content also showed a high concentration, especially at 1 and 4 hours after the injection. The salivary glands took up radioactivity to a large extent. The activity in the exocrine part of the pancreas was fairly high and remained so up to 24 hours after the injection.

Lymphatic organs

The spleen the thymus and the lymphatic nodes took up some radioactivity In the spleen the radioactivity was mainly concentrated in some active spots at 24 hours after the injection (fig. 6).

High concentrations were found in the *kidney* and the *bladder* from 5 minutes to 4 hours after the injection.

The *Harder s gland* showed a fairly high accumulation of radioactivity

The *skeletal muscle* the *fat* and the *skeleton* showed a low uptake of radioactivity

The *bone marrow* showed a moderate uptake of radioactivity 5 and 20 minutes after the injection

Placenta and foetus

The penetration through the placenta to the foetus was studied in two pregnant mice killed 1 and 4 hours after the injection. The radioactivity passed the placental barrier but the concentration in the foetus was lower than in the mother No specific localization in the foetus was observed (fig. 7).

Discussion

Investigations by LARSSON (1965), unpublished have shown that FG5111 is metabolized into several more polar compounds in rats and rabbits. It is therefore very likely that the autoradiograms show both the unchanged compound and its metabolites.

Our results show that FG5111 and its radioactive metabolites rapidly reach different parts of the CNS The highest uptake is seen in the thalamus and the hippocampus. The radioactivity leaves parts of the CNS fairly rapidly but remains to a considerable extent in the hippocampus at 1 and 4 hours after the injection, which corresponds to the duration of the pharmacological effects (CHRISTENSEN, HERNESTAM, LASSEN & STERNER 1965) The main excretory pathway seems to be in the urine, which is indicated by the present studies and the unpublished investigation by LARSSON Our results reveal that a considerable part of the radioactivity is excreted into the intestine via the bile Since LARSSON found only a few per cent excreted via the faeces, it is highly likely that reabsorption of the substance or its metabolites, or both, occurs from the intestine

Most of the radioactivity left the body within 24 hours. Radioactivity was then mainly observed in the liver the spleen, the intestines and the

kidney The spotty appearance of radioactivity in the liver and the spleen may indicate an uptake of the compound or its metabolites, or both, by the reticuloendothelial system. Approximately the same distribution picture was seen in the liver and the spleen after injection of tritiated dextran, which is known to be taken up by the reticuloendothelial system (HANNGREN HANSSON ULLBERG & ÅBERG 1959)

In many ways the distribution picture corresponded to what is found after injection of ^{35}S-chlorpromazine, which is distributed in the nervous system of mice in the same way as FG5111 (SJÖSTRAND CASSANO & HANSSON 1965) We noted the high uptake and retention of both compounds by the hippocampus. After chlorpromazine, however the radioactivity disappeared more quickly and more completely from the blood than after ^{14}C FG5111 The radioactivity stayed longer in the CNS after chlorpromazine injection than after FG5111 Both FG5111 and chlor promazine showed high radioactivity in the pituitary gland The high activity after FG5111 in the adrenal medulla was not seen after chlor promazine, which, on the other hand, showed high activity in the adrenal cortex

Summary

The bodily autoradiographic distribution of ^{14}C FG5111 (γ-(4-methyl-piperidino)-p-fluorobutyrophenone hydrochloride) after intravenous administration was studied in 7 male and 2 pregnant female mice

The cerebral cortex, hippocampus and thalamus showed high radioactivity from 5 minutes to 1 and 4 hours after injection The activity in pituitary and adrenal medulla was also high during the same period. Kidneys, liver and gastric glands excreted radioactive material. After 24 hours, most of the radioactivity was found in the liver and the intestines, but it was only a small part of the amount injected Because of the heavy excretion of radioactivity into gastric juice and bile, there was probably enterohepatic recirculation. The radioactivity passed the placental barrier in pregnant mice, but the concentration in the foetuses was lower than in the mothers. The radioactivity was evenly distributed among the foetal tissues.

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A Titrimetric Method for Separate Determination of Specific and Non-Specific Cholinesterase Activity in the Absence and Presence of Irreversible Inhibitors (Paroxan and DFP)

By

Jens Jensen-Holm

(Received April 7 1965)

As shown by ALLES & HAWES (1940), the cholinesterases of the organism is a mixture of two different main forms, best termed *specific* and *non-specific* cholinesterases. In erythrocytes the specific cholinesterase alone is present. In plasma a mixture of the two is usually present, though generally with preponderance of the non-specific component, judged by the methods employed so far (HAWKINS & MENDEL 1947 among others)

These enzymes (survey AUGUSTINSSON 1961) have been separate determinated in accordance with two different principles the aid of *specific substrates* or the aid of *specific inhibitors* Whereas acetyl-β-methylcholine (metacholine) is hydrolysed by the specific cholinesterase (AChE), benzoylcholine, butyrylcholine or proprionylcholine is hydrolysed by the non-specific (ChE) As pointed out in Augustinsson s survey however the specificity is not complete This may involve that, in the presence of excess of AChE, for instance, the value found for the activity of ChE will be a little too high, because AChE can also hydrolyse this substrate, though to a small extent only Use of specific inhibitors (introduced by ALDRIDGE 1953) has within recent years been regarded as the procedure of choice for *in vitro* measurements as well as for histochemical analyses. Here too the specificity is incomplete, however and the method is rather complex, as each sample must be subjected to not less than three analyses

(a) Measurement of [AChE] by adding a suitable inhibitor of ChE, (b) measurement of [ChE] by adding a suitable inhibitor of AChE, and (c) measurement of the combined activity without the inhibitors used for (a) or (b)

The result of (c) should at least be equivalent to the sum of the activities measured by (a) and (b). This sum can, however, be expected to be slightly lower because the inhibitors used are not sufficiently specific.

Measurements of cholinesterase activity in cases of poisoning by irreversible anticholinesterases require special steps to be taken in preparing the test mixture, partly to provide against a possible spontaneous reactivation and partly to prevent any possible continued inhibition after excision of the desired organ (BLABER & CREASEY 1959, JENSEN-HOLM 1960, SCHAUMANN 1960). As it is desirable not only to measure the combined activity but also to know the relative shares of [AChE] and [ChE] a method*) has been developed for which the above demands on the procedure can easily be met.

Method

The method is based in part on previous results for the course of the enzymatic activity at different acetylcholine concentrations. At concentrations exceeding about 10^{-3}M the specific cholinesterase activity is seen to decrease, whereas the non-specific activity increases (AUGUSTINSSON 1948, JENSEN-HOLM 1961). If these activities are known at two different substrate concentrations, it will be possible by a single mathematical calculation, to establish the relative [illegible] of [AChE] and [ChE].

Symbols used

AChI acetylcholine iodide
AChE specific cholinesterase
[AChE] specific cholinesterase activity (µmol [illegible] ml^{-1})
ChE *non-specific cholinesterase*
[ChE] non-specific cholinesterase activity (expressed as for [AChE])
C_s substrate concentration
pS neg. log of C_s

Cholinesterase activity was determined titrimetrically by means of Radiometer's "Titrator" and "Titrigraph" (JENSEN-HOLM, LAUSEN, MILTHERS & MØLLER 1959). By means of an external scale-expander (Radiometer Type PHN 630) the sensitivity of the apparatus was increased about 15 times, with a resulting considerable improvement in accuracy. The temperature (38°) and pH (7.40) were kept constant. The tissue concentration in the test mixture was kept at a low level, usually between 0.1 and 0.5% (wet weight). Any possible spontaneous acid or base production was carefully balanced by means of an adjustable, continuous addition of base or acid (JENSEN-HOLM 1961), which resulted in a slight base consumption (on the titrigraph). Then AChI was added to a concentration of 1 mM in the test mixture, and 2 – 3 minutes later the concentration was increased to 10 mM (see examples in fig. 1). The measured base consumption at the two concentrations of AChI was corrected partly for the base consumption before the addition of substrate and partly for the non-enzymatic substrate hydrolysis at the stated ACh concentrations. This was calculated to be 1.0[illegible] per hour of the amount present in the test mixture, irrespective of the concentrations used.

*) The method was described in a paper read before The Second International Pharmacological Meeting in Prague 1963.

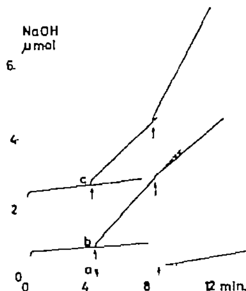


Fig 1 Examples of titrimetric recordings used for separating [AChE] from [ChE].

Abscissa Time in minutes.
Ordinate µmol of NaOH used in the titration.

a) The non-enzymatic hydrolysis f AChI at 10 mM t a volume of 50 ml saline.
b) In the presence of erythrocytes (guinea pig). The left part shows the slight base consumption before dding AChI The middle part. t 1 mM f AChI. The right part t 10 mM of AChI
c) In the presence of retroplacenta serum (Cohn fraction IV–6). The three steps of the curve as in (b).

Results

AChE alone

Erythrocytes from man and guinea pig were chosen as examples of occurrence of AChE alone. After centrifugation and washing several times with 0.9 / sodium chloride solution, haemolysis was produced by means of distilled water After the pH had been reduced to 6.5 the "shadows" were centrifuged to the bottom. These were washed several times at the pH stated (JORPES 1932 AUGUSTINSSON 1948) Then the enzyme activities of the "shadows" were determined at 1 and 10 mM of AChI. The ratio of the activities of the "shadows" at 10 and 1 mM proved to be identical with that found for plasma-free haemolysate and almost identical with that found for unwashed erythrocytes. The ratio of the activities at the highest and lowest substrate concentrations mentioned was calculated to be 0 645 ± 0 005 (s.e.m., n = 11) i. e. the activity at 10 mM was found to be about two-thirds of that at 1 mM The ratios found for human and guinea pig erythrocytes were identical

ChE alone

No organs, nor even any parts of organ nor plasma, contain non-specific cholinesterase alone (ChE) A suitable preparation was therefore required Measurements on plasma from normal humans gave ratios of activities at the highest and the lowest C_s, ranging from 1 70 to 1.80, against 1.35 to 1 45 in plasma from guinea pigs. The relative amount of [ChE] must therefore be greatest in human plasma Certain known factors were utilised to remove all AChE. ChE is completely destroyed at pH 2. At pH 4.5 AChE is destroyed (AUGUSTINSSON 1948) By temporary reduction of the pH it is possible to destroy AChE without an appreciable amount of ChE being lost. Thus, for instance, by reducing the pH to 3.9 for 90 seconds and then rapidly re adjusting it to 7 40, the ratio between the activities at 10 and 1 mM AChI was found to be increased to about 1 96–1.98 in both kinds of plasma (from human and guinea pig) With purified retroplacental serum (human Cohn's fraction IV–6 (AB KABI)), the ratio was calculated to be 1 975 ± 0 010 (s.e.m. n = 6), i. e the same ratio as for the acid treated plasma Acid treatment of the

Table I

Calculation of [AChE] and [ChE] in a random tissue specimen.

The analysis includes determination of the cholinesterase activity at 1 mM (A) and at 10 mM (B). The specific component will be reduced as indicated, and the non-specific one will be increased by elevating the substrate concentration from 1 to 10 mM The activity of AChE and ChE at 1 mM could now be found by solution of the two simple equations shown. In practice the calculation is made by determining the quotient (B/A) which is then found in the nomogram (fig 2), where the percentage distribution between [AChE] and [ChE] at 1 mM can be read directly

AChI	1 mM	10 mM
[AChE]	X	X × 0 645
[ChE]	Y	Y × 1.975
Sum	A = X + Y	B = X × 0 645 + Y × 1 975

A and B are known (the mixed activities measured at the two substrate concentrations used). X and Y can be found by solution of the equations

$$X = \frac{1.975A - B}{1.33} \quad \text{and} \quad Y = \frac{B - 0.645A}{1.33}$$

fraction of retroplacental serum mentioned had no effect in altering the ratio of activities at 10 and 1 mM AChI.

The procedure for calculating [AChE] and [ChE] in a given tissue sample is shown in table 1. After determining the ratio (B/A) between the activity at 10 mM (B) and 1 mM (A), this is found in the nomogram (fig. 2), where the percentage distribution between [AChE] (below the line) and [ChE] (above the line) can be read directly. The extreme points of the line, corresponding to the ratios 0.645 and 1.975 show the presence of [AChE] and [ChE] alone, respectively. That the curve is a straight line is evidenced from the simple equations given. The arrows seen in fig. 2 refer to ratios found for some organs, the subject of further comments in table 2.

Table 2

Determination of [AChE] and [ChE] at 1 and 10 mM AChI in different organs (from man and guinea-pig).

In the table are presented the results of determining [AChE] and [ChE] in erythrocytes and plasma from man and guinea pig. The distribution is shown at the two substrate concentrations used in the method described. The ratios (B/A, see table 1) are shown. For erythrocytes from man and guinea pig they were found to be the same and are grouped together.

For the ileum and brain from guinea pigs, the results are shown partly for untreated animals, partly after killing them with paroxan (diethyl-p-nitrophenylphosphate) given intravenously. The ratios for both organs are increased somewhat after the paroxan treatment. [AChE] must therefore be inhibited more than [ChE].

Tissue (normal)	n	ratio B/A ± s.e.m.[3]		[AChE] and [ChE] in % of total			
				1 mM ACh		10 mM ACh	
				[AChE]	[ChE]	[AChE]	[ChE]
a. erythrocytes[1,2]	11	0.645	0.0050	100	0	100	0
b. brain[2])[4]	5	0.739	0.0084	93.2	6.8	81.7	18.3
c. ileum[2]	5	1.302	0.0340	50.8	49.2	25.2	74.8
d. plasma[2]	3	1.356		46.6	53.4	22.[illegible]	77.8
e. plasma[1]	14	1.729	0.0065	18.6	81.4	6.9	93.1
f. fraction IV–6 or acid treated plasma	6	1.975	0.0104	0	100	0	100
After paroxan							
brain[2]	17	0.776	0.0287	90.0	10.0	75.2	24.8
ileum[2]	18	1.384	0.0490	44.5	55.5	20.8	79.2

[1]) human. [2]) guinea pig. [3]) standard error of the mean.
) cerebrum minus cerebellum and medulla oblongata.

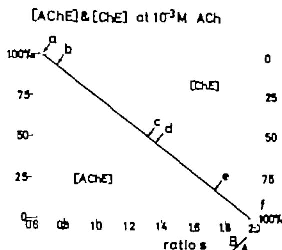


Fig. 2. Nomogram f r the distribution of specific and no -specific cholinesterase ctivity t 1 mM of AChI

Abscissa The ratios between the measured cholinesterase activities at 10 and 1 mM of AChI (B/A)

Ordinate [AChE] as percentage f the total chol nesterase activity measured t 1 mM f AChI

After determin g the ratio (B/A) described, the corresponding point on the nomogram is fo nd. Below and bove will be found [AChE] and [ChE] as percentages of the total activity measured t 1 mM of AChI

The rrows t a, b, c, d, e nd f give examples f ratios given in table 2 (normal tissues).

The effect of DFP (diisopropyl-phosphorofluoridate) in vitro incubation (human plasma and erythrocytes)

DFP is known as an irreversible inhibitor which at low concentration will mainly inhibit the non-specific cholinesterase In some experiments *in vitro* DFP was used to show the effect on the ratio (B/A), for human plasma, in which we should expect the ratio to decrease, in human erythrocytes, in which the ratio should be unaltered and finally for a mixture of the two in which again we would see a decreasing ratio The results are presented in table 3 DFP was used at concentrations from 2 to 20 ng/ml made from a stock solution of 10 mg/ml in propylene glycol diluted 1 1000 with distilled water The reaction volume was 50 ml time of incubation was 50 min., at 38 and reaction neutral The pH was taken to 7 40 10 minutes before addition of substrate, and the titration procedure was started

From table 3 it is seen that for plasma the ratio decreased from 1 75 to 1.37 Further decrease was impossible to demonstrate, because the activity measured would then be too low for exact calculation The inhibition of [ChE] is more pronounced than that of [AChE].

Table 3

The quotient (B/A) of human plasma (1), human erythrocytes (2), and f mixture of the two (3) at increasing concentration of DFP (di-isopropyl phosphorofluoridate).

The plasma nd erythrocytes used originated from the same normal person. DFP was added to the concentrations set out. Incubation at pH 7.40 was stopped after 50 minutes by the addition of AChI (final concentrations 1 mM and 10 mM). The activities at the higher and lower substrate concentrations were measured nd the ratio between them (B/A) was found. [AChE] and [ChE] were calculated as i table 2. For better comparison, the activities (t 1 mM) are expressed as

(1) Plasma per 200 µl
(2) Erythrocytes per 60 µl
(3) The mixture of the two per 200 µl plasma and 60 µl f erythrocytes.

	DFP µg/ml	Incubation (min.)	Ratio B/A	[AChE]	(%)	[ChE]	(%)
(1) µl of plasm							
400	0		1 748	0.0756	(100)	0.369	(100)
400	0		1 758	0 0732		0.373	
400	2	50	1 682	0 0572	(76.9)	0.203	(54 6)
400	5	50	1 574	0.0390	(52.4)	0.091	(24.5)
400	10	50	1 375	0 0154	(20.7)	0.016	(4 4)
(2) µl of erythr							
160	0		0.665	0 833	(100)		
160	2	40	0.690	0.728	(87 4)		
160	10	50	0.675	0.388	(46.5)		
160	20	40	0.675	0 287	(34.3)		
(3) plasma 200 µl + erythr 60 µl	0		1.071	0 899	(100)	0 404	(100)
	5	50	0.888	0.686	(79 8)	0.156	(38.6)
–	10	50	0.753	0.584	(68.0)	0.056	(13 8)
	20	50	0 671	0.416	(48.4)	0 009	(2.2)

On using erythrocytes alone, the ratio was rather unchanged. This means that we here only have AChE, and that the degree of inhibition at 1 and 10 mM of AChI is identical.

In the mixture of plasma and erythrocytes the ratio again decreased with increasing concentration of DFP from 1.07 in the control to 0 67 at the highest concentration used. At the ratio 0 67 the [AChE] was reduced by about 50 % and [ChE] was reduced by 98 %

Paroxan in guinea pigs

In guinea pigs poisoned by paroxan (diethyl p-nitrophenyl phosphate, E-600) (JENSEN-HOLM 1965) the ratio for the ileum (table 2) was found to have risen from 1.30 in the control material to an average of 1.38 Maximally it was found to be 1 70 In the brain the ratio rose from 0.74 to 0.78 (maximally 1 09) The groups of animals numbered 18 and 17 All the analyses showed the cholinesterase activity to be reduced The finding of the somewhat increased ratios for both organs probably signifies that the inhibition was most pronounced for [AChE] (cf ALDRIDGE 1950 BILLEWICZ-STANKIEWICZ *et al* 1954 HEATH 1961)

Discussion

The titrimetric method described above for the separate determination of [AChE] and [ChE] in the same vessel is simple and therefore offers certain advantages. On the other hand, it also has its limitations, being, among other things, inapplicable in the presence of reversible inhibitors, because these, by competing with acetylcholine, will have an unpredictable influence on the results Use of the method as described also presupposes that the apparature affords a possibility of adding acetylcholine by two stages and preferably that there is connection to a recorder Furthermore, an exact knowledge is required of the enzymatic activities of AChE and ChE at the substrate concentrations chosen In the case of very pronounced inhibition it is a considerable advantage to increase the sensitivity as described above, by attaching an external scale-expander

While, as stated, the method is unsuitable in the presence of reversible inhibitors, it is particularly suitable where irreversible inhibitors are concerned Using the dilution of tissue samples prescribed and the fairly rapid addition of acetylcholine (cf SCHAUMANN 1960) no further inhibition nor measurable reactivation of previously inhibited enzyme will take place during the analysis A rise of the acetylcholine concentration from 1 to 10 mM has no influence on the degree of inhibition of [AChE] or of [ChE] It must be considered an advantage that by the procedure described above the enzymatic action is expressed directly by the ability to hydrolyse acetylcholine, which is the physiological substrate Although the analytic precision is high, the determination of [ChE] is admittedly less accurate in cases with very low quotients (i e. approximating 0 645). Conversely the accuracy of determination of [AChE] will be reduced at very high quotients, approximating 1 975 This is, however in both cases a natural consequence of the fact that the activities mentioned then are very low

To demonstrate the usefulness of the method, two series of experiments have been carried out. Plasma and erythrocytes were incubated *in vitro* with increasing amounts of DFP which is known as an inhibitor first of all of ChE. In plasma, which contains both kinds of cholinesterases, the quotient is reduced with increasing concentration of DFP from about 1.75 to 1.38 showing that ChE is inhibited more than AChE. In the case of erythrocytes, where AChE alone is present, the increasing inhibition did not influence the quotient. This means that the inhibition degree at the two substrate concentrations used is the same. In the mixture of the two systems, the quotient again is reduced with increasing inhibition as was expected. From table 3 is seen that AChE is inhibited to a smaller extent than ChE, but the results could not be directly compared, because the amount of tissues used in the three series could not be the same. The behaviour of the specific cholinesterase, however, seems to be the same, showing that AChE is contained also in human plasma. A direct comparison could have been done if a great surplus of DFP was used, and the incubation should then be stopped after a few minutes. But such a surplus might possibly compete with acetylcholine.

Paroxan is known to inhibit AChE slightly better than ChE. In experiments on guinea pigs given paroxan intravenously at different rates of infusion until the animals were killed, the [AChE] and [ChE] in the brain and lower parts of ileum was determinated by the method mentioned. On an average, [AChE] was inhibited slightly more than [ChE], and as a consequence the quotient was elevated somewhat. In case of brain, the quotient rose from 0.74 to 0.78 and in ileum the quotient was increased from 1.30 to 1.38. The total activity at the moment of death, measured at 1 mM of AChI was found to be 7.3 / (brain) and 23.1 / (ileum).

In the two series carried out, we found that the quotient in the presence of DFP was reduced (or in the case of erythrocytes unchanged), and in the presence of paroxan the quotients for the brain and ileum both were increased. These results could be taken as a proof for the validity of the procedure.

We are not yet able to settle the exact number of active, enzymatic centres, but are obliged to express their action by the ability to hydrolyse an existing substrate, among others. The absolute values as well as the percentage distribution of the activity between AChE and ChE depend on the substrate concentration chosen.

At 6×10^{-2}M, [AChE] in human plasma constitutes 1.5 / of the combined activity measured. It is interesting to compare this result with that reported by HAWKINS & MENDEL (1947). By converting the figures given there based on measurement using acetylcholine and metacholine, into values expressed by ACh alone, we find that [AChE] constitutes

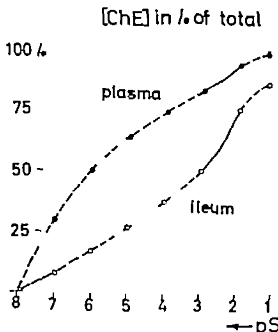


Fig. 3 The course of the distribution of [AChE] and [ChE] in huma plasma and in guinea pig ileum, at different concentrations of AChI

Abscissa pS = the negative log of C_s (the concentration f AChI)
Ordinate [ChE] as percentage of the total activity measured at different concentrations of AChI
Upper curve Human plasma (mea of 14 normal persons)
Lower curve Guinea pig ileum (mean of 5 animals)
The curves show percentage distributions of [AChE] nd [ChE] t different substrate concentrations. The relative sh re of [ChE] is decreased t decreasing substrate concentrations f ACh. The broken lines re extrapolations, made partly on the basis of the results obtained by JENSEN-HOLM (1961) who determined the course f the activity of erythrocytes nd of plasma (human) at various substrate concentrations. The share of [AChE] in plasma ere then subtracted from the plasma activity measured t the different substrat concentrations. I the ileum the distributions t 1 and 10 mM were directly determined The broken lines originates from calculations based as mentioned bove.

about 1 6 / of the total activity measured at 6 × 10 ² M ACh (cf DAVIES & OJHA 1950).

In fig. 3 is shown the percentage distribution at different substrate concentrations, illustrated by two examples human plasma, and ileum from guinea pig. The plotting of the curves are based on a knowledge of the course of [AChE] and [ChE] at different substrate concentrations (JENSEN-HOLM 1961) with a necessary correction for the share of [AChE] in the cholinesterase activity of human plasma, the influence of which had

been estimated too low in the reported study In the case of plasma, [ChE] at high substrate concentration was found to be almost equivalent to the total activity measured. At decreasing C_s the percentage share was, however calculated to be very considerably reduced Similar estimated conditions were noticed in the case of the ileum. If the extrapolations performed are correct, the curves suggest that ChE at low (physiological) concentration of ACh has no or very little acetylcholine-hydrolysing action This hypothesis is in fair agreement with results achieved by HAWKINS & GUNTHER (1946) HAWKINS & MENDEL (1947) SHELLEY (1955) and in part also with those of MUNKNER *et al* (1961) and JAMIESON (1963) These workers incline to the view that ChE hardly has any (significant) physiological or pharmacologic function as an acetylcholine-hydrolysing enzyme.

Summary

A simple titrimetric method has been described for simultaneous determination of specific and non-specific cholinesterase activity in the same test mixture with acetylcholine as the only substrate, successively at the two concentrations of 1 and 10 mM The ratios between the activities at the highest and the lowest concentrations were calculated as 0 645 for [AChE] and 1 975 for [ChE] With simultaneous occurrence of the two enzymes, their relative shares in the activity can easily be calculated.

The method described is sensitive It is suitable not only for normal organs, but also in the presence of irreversible cholinesterase inhibitors, but not in the presence of reversible inhibitors. DFP and paroxan, as examples of irreversible inhibitors, were used to demonstrate the validity of the method.

It has been concluded from the present and previous results that the non-specific cholinesterase (ChE) has possibly no significant acetylcholine-hydrolysing action *in vivo*

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Respiratory Changes in Guinea-Pigs Poisoned by Intravenous Paroxan – With a New Method for Separated Electronic Registration of Inspiratory and Expiratory Volumes

By

Jens Jensen-Holm

(Received May 3 1965)

Numerous biological experiments include measurements of respiration, whether from a wish to study respiratory functions or merely to check whether respiration is normal during an experiment. As a rule the classical method employed consists in recording by means of Marey's tambour By this procedure it is probably a combination of the volume and the flow rate of the tidal air that is measured.

With the introduction of an electronic apparatus it became possible to express the respiratory functions more accurately by means of physical units. For instance, we can measure the rate of the air flow (in millilitres per second), the volumes of expired and inspired air (in millilitres), as well as the respiratory rate (in cycles per minute), the movements of the thorax, and the fluctuations in intrapleural pressure. Each of the functions mentioned can be recorded by suitable electronic equipment (JORGENSEN 1964). As rate meters there can, for instance, be used an integrated impulse tachometer or a digital tachometer (which indicate the frequency by measuring the interval between two successive impulses)

The system employed for our study (see fig. 1) is in the main well-known in principle and was originally introduced by FLEISH (1925 & 1935), who described the pneumotachograph. His system consisted of a differential pressure manometer for measuring the difference between the pressures in two different sections of a specially constructed tube, through which the in-going and out going air were ensured laminar flow The faster the air passes through the tube, the greater is the difference in the pressures recorded. The system was improved by FLEISH (1956),

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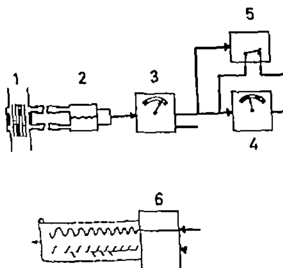


Fig. 1 *The experimental setup for determining respiratory function.*

1) *Pneumotachograph* (A. Fleisch) connected to a
2) *Low pressure differential transducer* (Statham, model PM 197), by which the pressure differences originated from the pneumotachograph are transformed into electric forces, amplified by an
3) *Amplifier* (Simonsen & Weel, model A 59). From this amplifier are connections to the writer (6) and to an
4) *Integration amplifier* (Simonsen & Weel, model A 59). Between the in- and output is placed an
5) *Automatic (interphasic) zero adjuster* (Simonsen & Weel, model AZC-64). The integration amplifier is connected to
6) *Mingograph ink writer* (Elema-Schönander model TLS- 8). Recordings are shown to the left of the writer

By (3) the respiratory air speeds are recorded. By the integration amplifier (4) these are transformed into air volumes. By means of (5) the volumes for inspiration and expiration are separated and the amplitudes are placed above and beneath the common zero line, as illustrated in fig. 2 and 3.

always becomes zero. It takes 5 msec t attain the utomatic zero level. When the relay switch then opens, the amplifier can integrate gain. The pparatus further affords the possibility of letting the zero adjuster remain activated throughout the expiratory or inspiratory phase, so that the amplifier for integration shows zero voltage during the phase involved. This allows tracing of the volume curve for the other phase alone.

The zero adjuster described has been constructed by the firm Simonsen & Weel (Model AZC-64. AZC = utomatic zero control). The following specifications ar required: Sensitivity 10 mV p-p. Input impedance 10 kilo ohm. Output impedance 100 ohm. Time of short circuit 5 msec. or half period of signal. The apparatus is able to follow rates of up to about 30 Hz.

6. *Mingograph ink writer* (Elema-Schönander Model TLS-8), provided with 8 galvanometers, of which 6 are used f recording various functions and two are intended for marking time and events. During our experiments the paper was advanced at a rate of

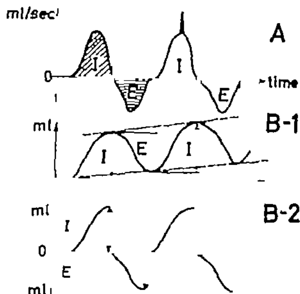


Fig. 2. *Determination of respiratory volumes by integration of the air speed during the respiratory cycles without and by use of the automatic (interphasic) zero adjuster shown in schematic way*

Upper curve. Respiration rate expressed in ml air/sec. (ordinate). The abscissa is the time course. The hatched areas are inspiration volume (I) or expiration volume (E).

Medium curve. The respiratory volumes (ml air) determined by integration without the use of the automatic zero adjuster. The volumes corresponding to the areas mentioned above are shown. Because (I) is different from (E), the curve soon will extend outside the recording paper.

Lower curve. The respiratory volumes determined by integration and simultaneous use of the *automatic zero adjuster* which will split up the curve (from the medium curve) here as indicated. The inspiratory volumes are now placed upwards and the expiratory volumes downwards from a common base line (zero line). Every time one passes from the one to the opposite respiratory phase, the curve immediately is taken down to the zero line. The next phase will then be written in the other direction. The time of short circuit is 5 msec., because of this we see an indication of the base line being a broken line, useful in the readings of the results.

It is possible to get tracings for either the inspiratory or the expiratory volumes alone.

between 0.5 and 5 mm/sec. At the slowest rates it is generally necessary to use an *trilliser for adjusting the line width* to the desired galvanometers, for the purpose of transforming the otherwise continuous line of ink into a narrow bundle of ink drops, to avoid ink blots on the paper.

The automatic zero adjuster described is combined with an integrated impulse tachometer which can be used either for simultaneous measurement of the respiratory rate or in free position for an arbitrary rate measurement. The tachometer was not used in the experiments described below.

The automatic zero adjuster (5) in the system reported has not been described before in this form. It was used for separated electronic registration of inspired and expired air volumes. The value of this apparatus will be shown below.

By means of the amplifier (3) curve is traced giving linear expression of the rate of the air flow one way the other through the Fleish tube. If desired, the curve can easily be calibrated to ml/sec. No such calibration was performed in our experiments. The course of the curve for respiratio is shown in fig. 2, A. The space between the traced curve and a base line, corresponding to an ir flow rate of zero (i. e. no measureable difference in pressures) is equivalent to the total volume f air passed during the period concerned. A high air flow of short duration (as indicated to the right in fig. 2, A) need not cause any appreciable augmentation f the space mentioned, even though the maximum of the curve thereby rises considerably The air volume passed is calculated by measuring the space. This is most simply done by electronic integration. The result of such an integration is seen in fig. 2, B-1 where the rising sections of the curve (i. e. the difference in height between the lowest and the next highest section), fo instance, equal the volumes of inspired ir The descending sections then correspond to the inspired volumes. The maxima and minima of the curve B-1 are situated at time levels corresponding to course f the respiratory rate (curve A) passing zero. It is seen that the curve B-1 is on its way out of the record space. The curve can be brought back gain by setting the amplifier for integration at "off" for moment. This manoeuvre can with advantage be executed automat ically t the conclusion of each phase by the automatic zero djuster described under (5).

A. Measurement of the inspired and expired air olumes by electronic integratio and use of the automatic zero adjuster

The principle of this rrangement has been described bove. The result of the co-operatio of the zero adjuster is shown in fig. 2, curve B-2. The ascending and descending sections seen in curve B-1 are here separated, so that each phase always begins at commo base r zero line For instance, the ascending sections represent the volumes f inspired air and the descending the volumes of expired ir The period for the utomatic adjust ment t zero level being, as stated, 5 msec., a correspondingly short curve section will accordingly be set out. A collection of such curve sections will now constitute a distinct base line, from which the in-going and out-going volumes of air can readily be estimated or measured. It is evident that drifting f the integration curve out of the record space now is voided.

The experiment carried out to demonstrate the usefulness of the pparatus i described below

The pneumotachograph was connected with syringe, whose piston was passed certain distance forwards nd backwards, corresponding to for instance 10 ml, imitating inspiratory and expiratory volumes. The speed at which the piston was moved varied. The results are shown in fig. 3 (and *b*). I both 3a and 3b the pper curve is a measure of the rate at which the air has passed through the Fleish tube, e of the speed at which the piston has been moved. This speed can be calibrated in ml/sec. The lower curve illustrates the courses of the volumes passed, traced by means of the amplifier for integration, with the zero adjuster ttached. In accordance with the arrangement of the experiment, the deflections are seen to be f the same size whether the piston was moved slowly or quickly The upward deflections correspond t passing of the piston in one direction and the downward deflections in the opposite direction It is also seen how if desired, one can record the deflections in the one direction alone. The ordinate here expressed in ml and can be calibrated by letting the syringe yield 2–4–6–8 and 10 ml at the time. By such calibration there was, as might be expected, found proportionality between the volumes used and the deflections obtained, provided the limits characteristic of the pneumotachograph, the transducer or the ink-writer were not exceeded.

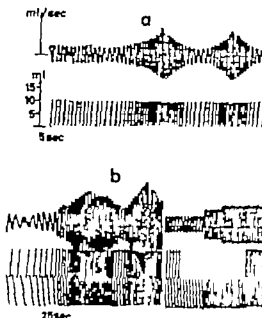


Fig. 3 *Determination of air volumes, originated from a syringe pushed to and fro, by electronic integration and use of the automatic (interphasic) zero adjuster*

The curves here shown represent the same as indicated by the upper and lower curves from fig. 2. The rate of the air passing the pneumotachograph is expressed in ml/sec. (*not calibrated here*), and the volumes, *after integration of the rate in time* are expressed in ml, in which the ordinate is calibrated (lower curve in *a* and in *b*).
The signals are made by means of a syringe, airfilled and connected to the pneumotachograph. The piston is moved at arious speeds, but the volume per cycle is kept constant. The pper curve (in or in *b*) will show the speed of the ai passing the tube. At high speeds the amplitudes are increased, and at low speeds they are decreased. Independent of the speed used, the olumes recorded in the lower curve (or in *b*) are identical, as would be expected. At extremely high speeds, a slight decrease in the vol me measured will be seen, because the range of the pneumotachogr ph is then exceeded.
The input and output of ir are seen to be separated in the curve representing the measured volumes. From the common base line the inputs are di ected upwards nd the outputs downwards. It is shown, too, that the zero adjuster affords the possibility of recording the one phase alone. The rest of paper then could be used for other purposes.

B. *Registration of respiratory volumes b electronic integration ithout sing the automatic zero adjuster*

In fig. 4 are shown the results f an ex ber experiment on guinea-pig given cont nous intravenous infusio of acetylcholine iodide. The upper curve showing the arterial pressure is of no interest in this connection. The middle curv how the rate of respiratory ir flow into and out of the pneumotachograph, connected t trachea. The bottom curve resulted from an electronic integration of the curve of the rate f the air flow thus ex pressing the volumes passed. It is seen, i fig. 4, that, despite accurate adjustment of the system before recording, the curve is soon on it w y out of th record pace, to hich it was brought back several times. The excursions f the vol me-curve traced were here

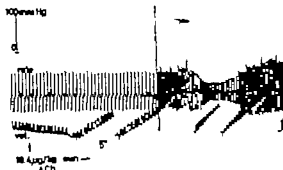


Fig. 4. *Determination of respiratory volumes by electronic integration of the air speeds, without use of the automatic (interphasic) zero adjuster during intravenous infusion of acetylcholine into guinea-pig*

The figure shows the effect of continuous i.v. infusion of acetylcholine into a guinea pig (urethane anaesthesia, 1.5 g/kg) on the respiration. *The upper curve* represent the arterial pressure in the carotis (measured by means of a Statham high pressure transducer Model P23AA). *The medium curve* gives the speeds of the expiration and inspiration, and *the lower curve* represents the volumes recorded after integration without using the automatic zero adjustor (earlier experiment). It is seen that this curve soon disappeared from the writing range, to which from time to time it was brought back manually. The amplitudes were kept small in order to keep the recordings so long as possible within the visible range. After 85 seconds the volumes were reduced, and the respiratory air rate was decreased also, after a transitory increase. The frequency was reduced.
In spite of the continued infusion of acetylcholine, the respiratory functions shown seem to be normalised again (possibly owing to adaption to a constant concentration of acetylcholine), whereas the blood pressure and the pulse frequency remained reduced.

kept small in an attempt to keep the curve within the record space as long as possible. It is evident that the zero adjuster described above is greatly missed.

Moreover the experiment showed that continued infusion of acetylcholine (19.4 µg/kg and minute) gradually reduced the respiratory functions. The air flow rate (after a short rise), the volumes passed and the respiratory frequency were all lowered. However despite continued administration of acetylcholine, these functions returned towards normal conditions. This indicates, perhaps, that a form of tolerance to a constant concentration of acetylcholine had very rapidly taken place (cf. KRIVOY & WILLS 1956, BRODEUR & DUBOIS 1964). The curve for the arterial blood pressure shows no such return to normal.

C. *Measurements of respiratory volumes based on the separated electronic registration of inspired and expired air volumes in guinea-pigs treated by continuous intravenous infusion of paraoxon* (diethyl-p-nitrophenyl phosphate). The rates of the respiratory air flow were measured also the expiratory frequency was found by simple counting of the cycles in short periods.

The experiments were conducted on ten guinea-pigs (average weight 570 g) under urethane anaesthesia (1.5 g/kg subcutaneously 90 minutes before). An inserted tracheal cannula was connected with the Fleish tube for measurements of respiration. The blood pressure was measured from the carotis connected to a high-pressure transducer (Statham, Model P 23 AA, maximal strain 750 mm Hg). The pulse rate was found by counting the

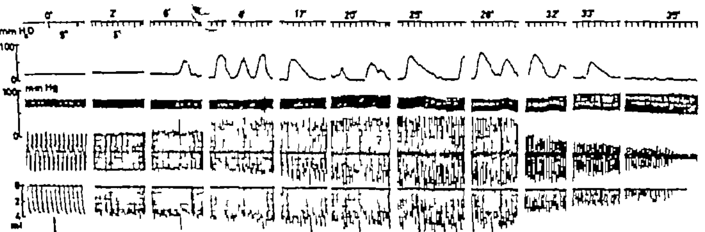


Fig. 5 *Determination of expiratory olumes by electronic integration of the air speeds, by use of the utomati (interphasic) zero adjuster during i travenous infusion of paroxan int a guinea-pig*

The figure show the effect of ntravenous paroxan (per minute 28.0 µg/kg) in guinea-pig (b. 19), on the intraluminal pressure in the ileum, the rterial pressure (carotis), the respiratory ai speeds and, first of all, the expiratory volumes, recorded after integration by the use f the automatic zero adjuster The inspiratory volumes are not shown. The intraluminal pressure in the ileum i recorded by means of thin-walled rubber balloon, inserted in the lumen and connected to low pressure transducer (Stath m, Model P23BB). The signal from the transducer is transferred to the writer after amplification. Ureth ne (1.5 g/kg) was used for naesthesia.

The curves shown are downwards for

1) the intraluminar pressure in the ileum (calibrated in mm H_2O)
2) the arterial pressure (mm Hg)
3) the respiratory ai speeds (ml/sec., but not calibrated here): the mplitudes pw rds represent the experatory phases, and downwards the inspiratory phases
4) the expiratory volume (in ml air; calibrated).

The results of the respiratory cha ges eceive comment in the text in connection with 9 other guinea-pigs to which, paroxan was given intravenously at different rates. A survey of these results is given in fig. 6 and fig. 7 The results from the experiment shown in his fig. are identical with the results show by the survey

cycles in short periods. A thin polyvinyl catheter was inserted in the jugular vein, proximally connected with an apparatus f r variable continuous infusion of paroxan. The speeds of infusion employed ranged between 16.1 and 263 μg/kg/minute. The experiments described formed part of another investigation (JUDEN-HOLM 1965) Further 20 mm long, thin-walled rubber balloon, measuring about 5 mm in diameter and filled with water was inserted in the ileum. The balloon was connected proximally with a low-pressure transducer (Statham, Model P 23 BB, with maximum strain 50 mm Hg). The recordings were performed as described above. I fig. 5 the results originating from guinea-pig no. 19 are given in extract. The uppermost f the four curves shows the fluctuations of the intraluminal pressure in the ileum intensified during the infusion. Next comes the curve for arterial pressure. The third curve illustrates the rate of the respiratory flow the upward deflections corresponding to the expiratory phases and the downwards to the inspiratory (cf LINDQUIST & SUNDWALL 1960). The lowest curve represents the volumes of expired air calculated by integration of the air flow rates in the phases of expiration, recorded by means of the zero djuster described. The instrumental possibility of leaving out one set f volumes (here the volumes of inspiration) is utilized.

Results and Discussion

Results of the experiments with intravenous paroxan

It is shown in fig. 5 (from guinea-pig no 19) that the maximum speed at which the expired air passed through the Fleish tube gradually increased considerably to fall again to values appreciably below the initial level towards the conclusion of the experiment. The inspired air behaved somewhat differently the passing speed of this having become slightly reduced at first. It then increased above the starting level, though not so much as that of the expired air The final fall was also less pronounced The small excursions seen at the extreme end of the curve were imparted by the heart action. For the measured volumes of expired air the single deflections increased somewhat during the first period, to decrease appreciably towards the end. The respiratory frequency was decreased

Survey of ten experiments

The results of the ten experiments performed were on an average approximately the same as shown by the example in fig. 5 no matter whether the speed of infusion was high or low i e whether the animals died after a shorter or longer period of infusion (the shortest and the longest period were found to 653 and 5563 *seconds respectively* The moment of death had been defined in advance to be *10 seconds after arrest of respiration*, estimated on the basis of the volume curve)

The average courses are shown in fig. 6 and fig. 7 In both figures the interval from beginning infusion to the animal's death has been set at 100 The various functions were then studied after passage of 0–10–20–30

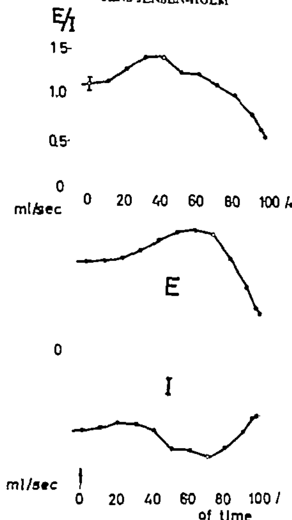


Fig. 6. *Determination of the respiratory air rates during infusion by paroxan to guinea-pigs.* The experimental conditions are detailed in the text to fig. 5 for one of the animals. In the other 9 the conditions were similar but the rates of infusion ranged from 16.1 to 263.0 µg/kg and min (of paroxan). The time from beginning the infusion until death ranged from 653 to 5565 seconds. The time of death was defined as cessation of respiration for 10 seconds, judged by the record of expiratory volumes.

The respiratory air rates were calculated as the means of at least 10 peak values. The readings were made before beginning and at 10–20–30 etc. % of the time from beginning to death.

The lower two curves illustrate the mean of the expiratory and inspiratory air rates during the experimental periods. *The expiratory air rate* (*E*) was increased after the start, and it reached a maximum at about 60 % of the time. *Thereafter it is decreased to less than 40 %* of the initial value. *The inspiratory air rate* (I) was slightly decreased in the first half of the time. Then it increased and maximum was reached at 70 % of the time. Lastly there was found a decrease again to a level just below the initial value.

We may therefore compare the respiratory condition just before death (with a prolonged expiration) with that of an attack of asthma bronchiale.

The upper curve shows the ratios between the expiratory and inspiratory air rates (E/I). It is seen, that E/I was elevated during the first 70 % of the time, with a maximum at about 35 %. In the final period E/I was decreased steeply

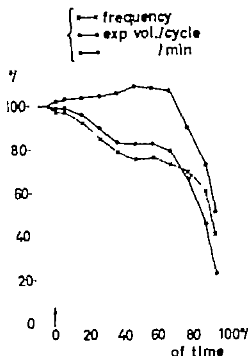


Fig. 7 *Determination of expiratory vol mes per cycle and per minute during intravenously infusion by paroxan into guinea-pigs*

The results originate from the survey of the 10 animals to which fig. 6 refers. For further details, see the text to fig. 6. The expiration volumes are the means of bout 20 readings on each animal. The means of the values from the 10 animals are given as percentages of the mean values just before beginning. The frequency is estimated from the curves as cycles per minute (the cycles read over 10–20 sec.). The expiratory volume per minute is the result of the first mentioned functions. All values re expressed as percentages of the initial values.

The initial values were

frequency 78.0 cycles per min.
expiratory volume per cycle 3.55 ml
expiratory volume per min. 277 ml.

The expirat ry singl volumes were increased during the most of the period. A maximum was reached in the middle of the time and was about plus 10%. Then the amplitudes decreased more and more. The frequency was reduced over the whole time. The curve shown seems to indicate two steps in this decrease. The final reduction goes on rapidly The expiratory volume per minute is reduced also in two steps. At the first step some compensation was brought bout by the increase in the single volumes of expiration. The second fall is accelerated because then the single expiratory volumes were decreased more and more.

etc. per cent of this period. Fig. 6 shows the average courses for the flow rates of expired and inspired air. The relation between these rates is also shown. It is seen that the maximum flow rate of the expired air during the experiments, both the absolute rate and that measured in relation to the rate of the inspired air flow, increased considerably during most of the period, with a subsequent steady steeper fall to less than the half the initial values. The conditions were otherwise identical with those illustrated in fig. 5. During the period immidiately before the animal's death, a prolonged expiration was found, and at the same time the phase of inspiration was of shorter duration, giving greater deflections. The condition therefore is comparable to an attack of bronchial asthma.

In fig. 7 are shown the average courses for *the single expiratory volumes, the expiratory minute volume* and *the respiratory frequency*. The expiratory *single volumes increased by up to 10% above the initial level, to fall* steeply during the last quarter of the period. The respiratory frequency fell gradually during the entire course. The single volumes having increased somewhat during the first three quarters of the experiments, the *fall in expiratory minute volume was less pronounced than the fall in* frequency. Towards the end of the period, when the single volumes also fell considerably, the decrease in expiratory minute volume consequently became more pronounced than that in frequency.

The average courses of the changes described show that an altered respiratory function was found immediately after the infusion had been begun. In another study (Jensen-Holm 1965) it was shown, in relation to these experiments, that the specific cholinesterase activity at the moment of the death had fallen to an average of 11.7% (brain) and 22.3% (ileum) of those in a control series. The corresponding figures for the non-specific cholinesterase activity were 10.1 and 30.6% respectively. Immediately after beginning the infusion the cholinesterase activity could not possibly be inhibited to any great extent. Even a slightly reduced cholinesterase activity must accordingly have been able to produce the pharmacological respiratory changes recorded.

Summary

1) An electronic equipment is described for the measuring of

a) the respiratory air flow rates,

b) the inspired and expired single volumes, recorded separately and

c) the respiratory frequency (or rates of other functions).

In connection with (b) the *automatic (interphasic) zero adjuster* is described. By means of this volumes are recorded from a common base

line If needed, the apparatus makes it possible to record one of these volumes alone.

2) In guinea-pigs, killed by infusing paroxan (an irreversible choline sterase inhibitor) at various rates, respiratory functions were followed
   a) *The rate of expired air flow* was increased during the first 80 % of the mean period until death, and then it decreased to below 40 % of the values at start.
   b) *The rate of inspired air flow* was, after a slight decrease, increased also In the last 10 % of the time it was decreased to just below the level at start.
   c) *The expired volume per cycle* was increased during the first three quarters of the period and then decreased steeply
   d) *the expiratory volume per minute* decreased throughout the ex perimental period, and the same was true of
   e) *the respiratory frequency*

   It is pointed out that these respiratory changes took place soon after beginning the paroxan infusion.

Acknowledgement

The author wishes to thank SIMONSEN & WEEL. Special thanks are due to Mr A JARLOV civil engineer who constructed the *automatic (inter phasic) zero adjuster* on the basis of requirements laid down for the apparatus.

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Acta pharmacol. et toxicol 1965 23 98–102

From the Department of Physiology Royal Veterinary College,
University of London.

The Toxicity of Some Halogenated Fatty Acids and Their Derivatives

By

N. Le Poidevin

(Received April 8 1965)

The fluorinated fatty acids show a mode of toxic action different from that of the other halogenated fatty acids (PATTISON 1959) In this study the term 'halogen is therefore taken to exclude fluorine Some of these halogenated fatty acids are known to be phytotoxic (POIGNANT & RICHARD 1957) and some toxic to stock (DALGAARD-MIKKELSEN & POULSEN 1962) The toxicities of these compounds are of interest, since they are employed in agriculture as herbicides and are also used as food preservatives. The only systematic study of their toxicity has been made by MORRISON (1946), who investigated the effects of a few of them on mice Previously he had studied their effects on yeast respiration (MORRISON 1943)

In our studies an attempt was made to relate the toxicity of monohalogenated fatty acids and their derivatives to their molecular structure, with a view to deciding whether or not the toxicity is related to the lability of the halogen and its ability to combine with thiol groups.

Experimental Methods

The acids and esters not a ailable from commercial sources were synthesised by standard preparative methods. Before injection, the acids were neutralised with sodium carbonate solution, and the esters were dissolved in 3 % (/v) cellosolve Injection f 0.5 ml quantities int white female mice of 25 g body weight was intraperitoneal. Dose levels were from 1000 mg/kg downwards, in twofold serial dilution. At least three mice were used for each dose level. They were given free access to food and water Those dying within seven days from injection were held to ha been killed by the substance injected. The LD50 alues wer calculated by the method of I win & CHEESEMAN (1939).

Results

The results are given in tables 1 2 & 3

Table 1

LD50 values of some halogenated fatty acids for mice. Intraperitoneal (mg/kg)

Acid	Chloro-derivative	Bromo-derivative	Iodo-derivative
2 Haloacetic	169	85	45
2 Halopropionic	>1000	200	283
3-Halopropionic	>1000	>1000	812
2-Halobutyric	>1000	325	211
3-Halobutyric	>1000	>1000	>1000
4-Halobutyric	>1000	>1000	>1000
iso-Halobutyric	–	>1000	707
2 Halovaleric	–	635	–
2 Haloisovaleric		793	–
2 Halohexanoic	–	354	566
2 Haloheptanoic	–	354	–
2 Halooctanoic	–	280	–
2 Halononanoic	–	223	283
2 Halodecanoic	–	177	283
2 Haloeradecanoic	–	504	–
2-Halododecanoic		566	–

Table 2

LD50 values f the esters of some halogenated fatty cids for mice. Intraperitoneal (mg/kg)

	Chloro-derivative	Bromo-derivative	Iodo-derivative
Ethyl			
2 Haloacetate	223	63.5	71
2 Halopropionate	–	353	–
3-Halopropionate	–	>1000	–
2 Halobutyrate	1120	891	707
3-Halobutyrate	707	500	>1000
4-Halobutyrate	891	>1000	891
2 Haloisobutyrate		891	562
2 Halovalerate	–	707	–
2-Haloisovalerate	–	1120	–
2-Halohexanoate	–	707	–
2 Haloheptanoat		707	–
2 Halooctanoate	–	707	–
2-Halononanoate	–	>1000	–
-Halodecanoate	–	1120	–
2-Haloundecanoate	–	707	–

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In our studies an attempt was made to relate the toxicity of monohalogenated fatty acids and their derivatives to their molecular structure, with a view to deciding whether or not the toxicity is related to the lability of the halogen and its ability to combine with thiol groups.

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The acids and esters not available from commercial sources were synthesised by standard preparative methods. Before injection, the acids were neutralised with sodium carbonate solution, and the esters were dissolved in 3% (/v) cellosolve. Injection of 0.5 ml quantities into white female mice of 25 g body weight was intraperitoneal. *Dose levels were from* 1000 mg/kg downwards, in twofold serial dilution. At least three mice were used for each dose level. They were given free access to food and water Those dying within seven days from injection were held t have been killed by the substance injected. The LD50 values were calculated by the method f LITCHFIELD & CHEESEMAN (1939).

Results

The results are given in tables 1 2 & 3

would be expected to render the higher acids more toxic than bromodecanoic.

The higher iodo acids were not examined as thoroughly as their bromo analogues, as it was not possible to prepare pure samples of them Nevertheless the crude samples showed similar trends of toxicity They were less toxic than their bromo analogues, a feature that could be associated with a more rapid hydrolytic dehalogenation and supports the view that a combination with thiol groups is not involved. On the other hand, death ensued more rapidly from the iodo acids than from the bromo ones.

As the halogen atom of esters is more labile than that of the free acids, it might be expected that the esters would be more toxic This has been found *in vitro* (MACKWORTH 1948) The fact that the esters were less toxic could be explained by the fact that they hydrolyse more rapidly than the corresponding acids

A possible mode of action of the higher halogenated acids that would explain why there was a maximum toxicity at a particular chain length could take into account the fact that fatty acid oxidation is inhibited by some unsubstituted acids of specific chain lengths (HOCHSTER & QUASTEL 1963) and involves an inhibition of enzyme systems associated with fatty acid oxidation

The toxicity of bromopyruvic acid, which approached that of fluoracetic acid, deserves special mention. MAGER & BLANK (1954) showed that fluoropyruvic acid was considerably less toxic to mice than fluoroacetic acid. PETERS & HALL (1957) argued that the presence of two adjacent carbonyl groups renders the fluorine atom sufficiently labile to combine with thiol groups. As the extent of such a combination usually increases with the atomic number of the halogen, it would be expected that chloropyruvic and bromopyruvic acids would be more toxic. This was found to be so and bromopyruvic acid was the most toxic of the acids examined As methyl and ethyl bromopyruvates possess a similar toxicity and are hydrolysed almost instantaneously by water it is evident that this toxicity depends on the bromopyruvate ion and not on the undissociated molecule The fact that these compounds are toxic has special interest because they are being used in synthetic organic chemistry

Summary

The toxicity to mice of a number of monohalogenated fatty acids and their derivatives has been studied. The toxicity depends upon substitution of the halogen at the 2-carbon atom and increases with the atomic number of the halogen. The toxicity of the 2 bromo acids decreases from

bromoacetic to bromovaleric, but increases to a second maximum at bromodecanoic acid. The esters were less toxic than the acids. Bromopyruvic acid possessed a similar order of toxicity to fluoroacetic acid.

Acknowledgements

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From the Department of Pharmacology University of Göteborg, Sweden

Glycogenolysis and Lactic Acid Production by Isolated Diaphragm from Fasted and Fed Rats in Bicarbonate and Phosphate Buffers, with Special Reference to the Effect of Adrenaline

By

Nils Svedmyr

(Received April 24, 1965)

SHAW & STADIE (1957) found that insulin in the presence of glucose stimulated lactic acid production by isolated rat diaphragm in bicarbonate buffer but not in phosphate buffer The synthesis of glycogen, on the other hand, increased to the same extent in the two media. RANDLE & SMITH (1958) found that the rat diaphragm under anaerobic conditions utilized glucose in bicarbonate but not in phosphate buffer HERMAN & RAMEY (1960) observed, moreover that adrenaline depressed the uptake of glucose by rat diaphragm in phosphate but not in bicarbonate buffer

Adrenaline stimulates glycogenolysis and lactic acid production in striated muscle, probably by activating phosphorylase, an enzyme that intensifies the breakdown of glycogen into glucose 1 phosphate (SUTHERLAND & RALL 1960) The study described here was aimed at investigating these effects of adrenaline more closely and it seemed of interest to determine whether the action of adrenaline was the same in bicarbonate as in phosphate buffer

The experiments were performed on isolated rat diaphragm, a tissue in which RIESSER (1947) WALAAS & WALAAS (1950) and WALAAS (1955) had previously studied the glycogenolytic and lactic acid-stimulating effects of adrenaline The tests were made in the absence of glucose.

It was incidently found that spontaneous lactic acid production in diaphragms from fasted rats was appreciably greater than the loss of glycogen. This initiated a study of the carbohydrate metabolism in diaphragms from fed rats as well

Methods

Fasted rats. Diaphragms from three or four previously well-fed rats weighing 120–180 g were used in each run of experiments with bicarbonate or phosphate buffer medium. Different diaphragms were used for the bicarbonate and phosphate tests. The animals received no food for 20 hours and were killed by a blow on the head, after which their diaphragms were dissected out and immersed in the particular ice cold buffer solution to be used subsequently in the experiments. Each diaphragm was divided centrally one half being used as a control, and the other half for incubation with adrenaline. After 30 minutes preincubation at 37°C in 15 ml bicarbonate or phosphate buffer each preparation was divided once again. After careful drying on filter paper one part was frozen in difluordichlormethane (Frigen or Freon 1) containing carbon dioxide snow and then one third of it was taken for glycogen assay and the rest for lactic acid determination (basal values). In the tests with adrenaline the remaining quarters of the diaphragms were incubated for 15 minutes at 37 C in 15 ml buffer containing adrenaline at a concentration of 10^{-6}

Fed rats. In another series of experiments the spontaneous glycogenolytic rate and lactic acid production in bicarbonate and phosphate buffer media were studied in diaphragms from fed rats. In these tests pieces of the *same* diaphragm were incubated in bicarbonate or phosphate buffer media for 45 min. The tests were in other respect performed as described above.

On completion of incubation the muscle was assayed for glycogen and lactic acid, as described above, and the lactic acid contents of the incubation solutions were also determined. For calculation of the lactic acid production during incubation, the amount of this substance in the muscle at the beginning of the experiment was deducted from the amount at the end of the experiment together with that in the incubation solution. The degree of glycogenolysis was estimated from the differences in glycogen contents before and after incubation.

The bicarbonate buffer had the molar composition NaCl 0.12 KCl 0.0047 $CaCl_2$ 0.0025 $MgSO_4$ 0.0012 KH_2PO_4 0.0012 $NaHCO_3$ 0.025 When aerated with 95 % O_2 and 5 % CO_2 the solution assumed a pH of 7.4 The phosphate buffer was composed of NaCl 0.13 KCl 0.005 $CaCl_2$ 0.003 $MgSO_4$ 0.001 Na_2HPO_4 0.01 and HCl 0.002. Aerated with 100 % O_2, this solution had a pH of 7.4 Both buffer solutions were glucose free.

The glycogen content was determined by a previously reported method (LUNDHOLM & MOHME-LUNDHOLM 1957) in groups of three or four strips of diaphragm, totalling 0.1–0.15 g muscle tissue.

For determination of the lactic acid content of the muscle the frozen preparations, with total weight of 0.25–0.35 g, were immersed in 5 ml iced 10 % trichloracetic acid and then homogenized in an Ultra-Turrax apparatus (manufactured by JANKE & KUNKEL). The apparatus was rinsed with an additional 10 ml 10 % trichloracetic acid, the homogenate was centrifuged and the solution was then assayed for lactic acid by the procedure of FRIEDEMANN & GRAEser (1933) The lactic acid content of the incubation solution was also determined by the same method after addition of 15 ml 10 % trichloracetic acid

Results

Glycogenolysis and lactic acid production in diaphragms from fasted rats effects of adrenaline The results are detailed in tables 1 and 2. No unequivocal spontaneous glycogenolysis during the 15 minute incubation period was demonstrated in the control experiments with either bicarbo-

Table 1

The effect of adrenaline on glycogenolysis of isolated rat diaphragm from fasted rats in bicarbonate and phosphate buffer P = probability that the effect was due to chance. n = n mber of tests.

	Basal glycogen content mg/100 g	Change of glycogen content from basal value, mg/100 g/15 min. control	adrenaline 10^{-6}	Mean difference adrenaline-control in the same tests
Bicarbonate buffer n = 12	137 5 ± 17 1	+7.8 ± 12.8	−49 0 ± 11.3 P < 0.001	56.8 ± 9 0 P < 0.001
Phosphate buffer n = 9	126.4 ± 11.8	−7 1 ± 6.4	−30.9 ± 6.4 P < 0.01	23 8 ± 9.3 P < 0.05

Difference in adrenaline effects in bicarbonate and phosphate buffer 33.0 ± 12.9 P < 0.02.

Table 2

The effect of adrenaline on lactic acid production of isolated rat diaphragm from fasted rats in bicarbonat nd phosphate buffer

	Lactic acid production mg/100 g/15 min. control	adrenaline 10^{-6}	Mea increase of lactic acid produc tion after adrenaline in the same tests mg/100 g/15 min
Bicarbonate buffer = 12	20 3 ± 3 4	46.3 6 6	26.0 ± 5.4 P < 0 01
Phosphate buffer = 9	18.6 ± 4 7	34 0 ± 4 7	15.4 ± 6.7 P < 0.05

Difference between adrenaline effects in bicarbonate and phosphate buffer 10.6 ± 8.6.

nate or with phosphate buffer In these experiments, however appreciable lactic acid production was manifest in each of the incubation media. In bicarbonate buffer the lactic acid production exceeded the change of glycogen content in the same tests by 28 1 ± 8 1 mg/100 g/15 min (P < 0.02). It seemed likely that this lactic acid had been formed from some other source than glycogen.

Under the influence of adrenaline, glycogenolysis increased markedly in bicarbonate buffer and the glycogen loss and lactic acid production were of the same magnitude. Appreciable glycogenolysis was also demonstrated in phosphate buffer but the effect of adrenaline on the

Table 3

Spontaneous glycogenolysis and lactic acid production of isolated rat diaphragm from fed rats in bicarbonate and phosphate buffer Mean of 13 tests. Glycogenolysis and lactic acid productio in mg/100 g muscle/45 min.

Basal glycogen content	Decrease in glycogen content		Lactic acid production	
	bicarbonate buffer	phosphate buffer	bicarbonate buffer	phosphate buffer
766.1 ± 25 0	88.8 ± 15.0	89 9 ± 13.5	63 0 ± 9.0	57 2 ± 7.5

glycogen content was only about 40 / of that in bicarbonate buffer The difference was 33 0 ± 12.9 ($P < 0\ 02$) Hence adrenaline probably had a lesser glycogenolytic effect in phosphate than in bicarbonate buffer In both media, however it manifestly stimulated lactic acid production. Although the effect appeared somewhat greater in bicarbonate than in phosphate buffer the difference was not statistically significant.

Spontaneous glycogenolysis and lactic acid production in diaphragms from fed rats The smaller glycogenolytic effect of adrenaline in phosphate than in bicarbonate buffer may possibly be attributed to more rapid spontaneous glycogenolysis in the former To exclude this possibility spontaneous glycogenolysis and lactic acid production in bicarbonate and phosphate buffers were determined in different pieces of rat diaphragm from the same animal. As the glycogen content of the diaphragm may have a rate-limiting effect on spontaneous glycogenolysis, the experiments were performed on diaphragms from fed rats, which explains the higher basal glycogen content than in the previous set of tests. As may be seen from table 3 spontaneous glycogenolysis in these tests was the same in both phosphate and bicarbonate buffer The loss of glycogen was, moreover greater than the lactic acid production, so that the glycogen in diaphragms from fed rats probably constituted the substrate for lactic acid formation.

Discussion

Shaw & Stadie (1959) suggested that the enzyme phosphofructokinase had a decreased activity in phosphate buffer Murad *et al* (1962) observed that the stimulating effect of catecholamines on the formation of cyclic 3 5 AMP *in vitro* was less in phosphate buffer than in Tris buffer In my own experiments the glycogenolytic effect of adrenaline was smaller in phosphate buffer than in bicarbonate buffer The lactic acid stimulating

effect was the same in both. Neither spontaneous glycogenolysis nor lactic acid production was reduced in phosphate buffer These results may indicate that inhibition of the adrenaline effect was due to the fact that the activation of phosphorylase by adrenaline was less in phosphate than in bicarbonate buffer

In diaphragms from fasted rats the spontaneous lactic acid production was greater than the loss of glycogen, whereas in diaphragms from fed animals the glycogen loss was more than sufficient to provide for the lactic acid production. These tests seemed to demonstrate that diaphragms from fasted animals were able to produce lactic acid from some source other than glycogen.

Summary

In experiments on isolated diaphragms from fasted rats in glucose-free bicarbonate buffer lactic acid production was appreciably greater than the loss of glycogen that is, lactic acid was formed from some source other than glycogen In fed rats the degree of glycogenolysis was greater than the lactic acid production. After adrenaline the glycogenolysis and lactic acid production were of the same order of magnitude in diaphragms from fasted rats. The glycogenolytic effect of adrenaline was greater in glucose-free bicarbonate than in glucose free phosphate buffer No significant difference was found between the lactic acid stimulating effect of adrenaline in phosphate buffer and in bicarbonate buffer

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From the Research Laboratories of Ferrosan, Malmo and Copenhagen.

Pharmacological and Toxicological Studies on γ-(4-methylpiperidino)-p-fluorobutyrophenone (FG 5111) – a New Neuroleptic Agent

By

J A Christensen, S. Hernestam, J B. Lassen and N. Sterner

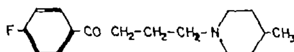
(Received February 12, 1965)

The introduction of chlorpromazine into psychiatric therapy (DELAY 1952) marked a great advance in psychopharmacology Since that time several phenothiazine and thiaxanthene derivatives have been introduced. In spite of the appearance of these new and often valuable agents, there still seems to be a need for new neuroleptics with fewer side effects or having a different chemical structure and so also another mechanism of action.

FLOREY (1956) reported the presence in the mammalian brain of a certain factor I which inhibits the generation of impulses by stretch receptor neurones in the crayfish. FLOREY & LENNAN (1956) showed this factor to have an inhibitory effect on central synaptic transmission in mammals. Investigations by ELLIOT & FLOREY (1956 1957) indicated that the active substance of factor I is γ-aminobutyric acid (GABA), and HAYASHI (1959) assumed that the real central inhibitory factor is γ-amino-β-hydroxybutyric acid (GABOB)

In the search for new psychotropic drugs related to GABA, we have synthesized and pharmacologically investigated a series of butyrophenone derivatives. One of the most active central depressants among these substances is FG 5111 JANSSEN *et al* (1959) have prepared several butyrophenone derivatives related to pethidine, of which some have been introduced into psychiatry The one best investigated clinically is haloperidol (fig. 1), which was introduced by DIVRY *et al* (1959)

Chemically FG 5111 is γ-(4-methylpiperidino)-p-fluorobutyrophenone hydrochloride (fig. 1) It forms colourless crystals, m.p. 209–211 soluble in polar solvents such as water ethanol and chloroform. It is light resistant, non-hygroscopic and bitter in taste.



FG 5111

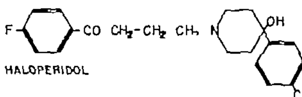


Fig. 1 Structural formulae of FG 5111 and haloperidol.

Studies of metabolism and excretion of ^{14}C-labelled FG 5111 are in progress The results obtained so far on rats and rabbits have shown that after administration of a single dose most of the radioactivity may be recovered within 24 hours in the urine and faeces (LARSSON unpublished results) After 72 hours, up to 70 % of the activity had been excreted. Only a few % of the dose, however seemed to be excreted in the unchanged form. In rat urine at least 10 different metabolites were present, the main one probably being hydroxylated in the benzene ring. In rabbits, on the other hand, the main metabolite was identified as p-fluorohippuric acid, but small quantities of other metabolites were also found

The distribution of radioactive material after i.v. injection of ^{14}C labelled FG 5111 was investigated autoradiographically by EINER JENSEN & HANSSON (1965) in pregnant and non-pregnant mice

Methods and Results

All the LD50 and ED50 values given below and the confidence limits (P = 0,05) were calculated by the method of LITCHFIELD & WILCOXON (1949)

Table 1

Prolongation of hexobarbital anaesthesia by administration of the barbiturate at different times after the test drugs.

Compound		Sleeping time in min. after				
		½ h	1 h	2 h	4 h	7 h
FG 5111 10 mg/kg	s.c.	30	17	18	9	8
Chlorpromazine 5 mg/kg	s.c.	30	16	26	9	7
Control (saline s.c.)		4	4	4	4	4

A. *Potentiation of anaesthesia*

Barbiturate anaesthesia.

Sleeping time was determined in mice after intravenous injection of hexobarbital 50 mg/kg. The sleeping times of mice, pretreated subcutaneously with the test compound (6 mice/dose) 30 min before the hexobarbital injection, were compared with those of untreated controls by the method of WINTER (1948) The ESD was the dose that prolonged the sleeping time to four times that of the controls. In other experiments the effect of a standard dose given at different times before the injection of hexobarbital was determined.

The ESD for barbiturate potentiation was 2.0 (1 6–2.5) mg/kg compared with 1 0 (0 8–1.3) for chlorpromazine and 5 (3.5–7.2) for haloperidol. The duration of the hexobarbital potentiation was about the same for FG 5111 and chlorpromazine (see table 1).

Ethanol anaesthesia.

The test drugs were administered orally (10 mice/dose) immediately before an i. p dose of 5 g/kg ethanol The sleeping times were determined by the method of FORNEY *et al* (1962)

10 mg/kg FG 5111 6 mg/kg chlorpromazine and 2 mg/kg haloperidol were the lowest doses to give a significant prolongation of ethanol anaesthesia (Student s test, p ≤ 0 05)

B *Effect on spontaneous activity*

Climbing test.

The method was described by KNEIP (1960) and SANDBERG (1959) Groups of 6 mice were put in a cage with a net ladder which permitted the animals to climb out of the cage normally all mice left the cage in a

Table 2

Mean effective doses (mg/kg s. c.) in tests on spontaneous activity

Compound	Climbing test ED50	Activity wheels ED50
FG 5111	1.2 (0.8–1.8)	2.6 (1.6–4.3)
Chlorpromazine	1.8 (1.1–2.9)	2.3 (1.6–3.1)
Haloperidol	1.5 (0.8–2.9)	0.3 (0.2–0.4)
Meprobamate	138 (125–152)	180
Chlordiazepoxide	19 (13–27)	60 (36–99)

few minutes (high exploring activity in a new environment). The test drugs were administered subcutaneously (6 animals/dose) 30 min. before the test. The ED50 was the dose that made 50 / of the animals remain in the cage for 10 min

Activity wheels

A special design of activity wheel, giving the animals free choice to enter was used (LJUNGBERG 1957 STRÖM 1964) One mouse was put in each cage with a revolving drum 15 min after a subcutaneous injection of the test substance, and the activity in drum revolutions per hour was recorded Half the animals were given physiological saline, the other half receiving the test substance. After three days the procedure was repeated with the groups interchanged, so that each animal acted as its own control. Individual quotients of activity and the average values of these quotients were calculated At each dosage 12 mice were employed. The ED50 was the dose giving a 50 / decrease in activity

As shown in table 2, FG 5111 chlorpromazine and haloperidol greatly reduced spontaneous activity in both tests. In the activity wheel test, however haloperidol was considerable more active than the other two drugs. Only relatively weak effects were exerted by meprobamate and chlordiazepoxide

C *Effect on motor coordination*

Rotating rod

The motor coordination of mice was determined by the rotating rod procedure (DUNHAM & MIYA 1957) The test substance were administered s. c., (6 mice/dose) 30 min before challenging the ability of the mice to remain on a rotating wooden rod (diameter 4 5 cm 1 rev /min) for one min. The ED50 was the dose that caused 50 / of the mice to fall off the rod.

Table 3

ED50 (mg/kg s.c.) in tests on motor coordination.

Compound	Rotating rod	Inclined plane
FG 5111	31 (23–41)	2.5 (1.6–3.9)
Chlorpromazine	20 (15–26)	1.3 (1.0–1.5)
Haloperidol	7 (5–10)	1.1 (0.7–1.8)
Meprobamate	90 (74–110)	>140
Chlordiazepoxide	35 (28–44)	>120

Inclined plane.

The test of NIESCHULZ *et al* (1955) was used. A mouse was placed on a smooth metal plate at an angle of 30° to the horizontal plane. Normal mice could move on the plate without difficulty but ataxic mice slid off. The test drug was injected subcutaneously (6 mice/dose) 30 min. before the test. The ED50 was the dose causing 50% of the animals to slide off the plate.

The ataxia-inducing effects of the test compounds are shown in table 3 for which it is apparent that FG 5111 held an intermediate position. Haloperidol was most and meprobamate least active.

D. *Effect on conditioned avoidance response*

The apparatus of JACOBSEN & SONNE (1955) was used. As described by COOK *et al* (1957) rats were trained to avoid an electric shock (unconditioned stimulus – US) by escaping from one compartment to the other when a buzzer signal (conditioned stimulus – CS) was given. When they responded to the CS, a conditioned response (CR) was considered to have been elicited.

The test drug was given subcutaneously to 10 rats on each dose. After 2 hours the rats were placed in the cage, and the reactions to CS and US were observed. If the animals failed to escape after CS but reacted to the US, a specific blocking action on the CR was considered to have been obtained. The ED50 was the dose at which this occurred in 50% of the animals.

This reflex was specifically blocked by FG 5111, chlorpromazine and haloperidol. The ED50 values were 6.6 (4.9–8.7), 2.0 (1.1–3.5) and 0.5 (0.3–0.8) kg/kg, respectively showing that FG 5111 had the weakest and haloperidol the strongest activity of the three drugs. Meprobamate

Table 4

Effect on body temperature in mice (6 animals/dose).

Compound	Route	Dose mg/kg	Fall in temp. Centigrad	Max. effect at min.
FG 5111	s. c.	2.5	2.0	60
–	s. c.	5 0	3 1	60
–	s. c.	10 0	4 0	60
–	s. c.	20.0	5 1	120
–	p. o.	25 0	1 5	30
–	p. o.	50.0	4.0	60
–	p. o.	100 0	6.8	90
Chlorpromazine	s. c.	1.25	1.8	120
–	s. c.	2.5	2.9	150
–	s. c.	5.0	5.3	180
Haloperidol	s. c.	5.0	2.5	120
–	s. c	10 0	5 0	120

was not active at the dose tested, and chlordiazepoxide had a weak unspecific action (ED50 ∼ 20 mg/kg, on which dose the animals were ataxic)

E. *Effect on body temperature*

The rectal temperature of the mice was determined with a thermocouple, readings being taken every 30 min The test compound were injected subcutaneously (6 mice/dose) The experiments, each including 6 experimental and 6 control mice, were carried out at constant room temperature (22°).

FG 5111 chlorpromazine and haloperidol exerted a marked hypothermic effect upon the mice. Table 4 lists the results of temperature experiments, and fig. 2 shows the temperature-curves resulting from giving 5 mg/kg subcutaneously of the three substances. The temperature-reducing effect of FG 5111 and haloperidol appeared to be about the same, but the effect of haloperidol was longer lasting than the effect of FG 5111 Of the substances tested chlorpromazine showed the strongest and most prolonged hypothermic effect.

F *Analgesic activity*

Writhing test.

Intraperitoneal injection of irritant substances into mice has been shown to cause a special syndrome, which may be abolished by analgesics

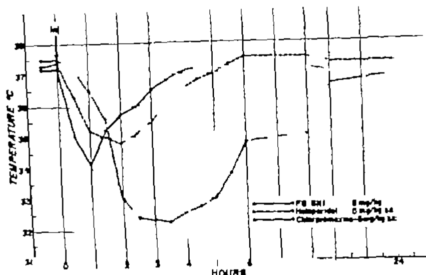


Fig. 2. Hypothermic effect in mice (6 animals/dose).

(SIEGMUND *et al* 1957 ECKHARDT *et al* 1958). As irritant acetic acid (15 ml/kg of a 0,5 / solution) was used In 100 / of the control animals this solution provoked a writhing syndrome. The test substance was injected subcutaneously 20 min. before the acetic acid (6 mice/dose) 10 min. later the animals were observed for the writhing syndrome during 5 min. The ED50 was the dose at which the writhing syndrome was abolished in 50 / of the animals. This test was also employed to ascertain whether the analgesic effect of substance 5111 was antagonized by nalorphine with morphine as reference substance.

Hot plate method.

Thermal pain stimuli have been utilized in different studies of analgesic activity The method of WOOLFE & MACDONALD (1944) was used. The test substance was administered subcutaneously (6 mice/dose) 30 min. before placing the animals on an iron plate thermostatically controlled at 55° The control mice showed active signs of discomfort. The ED50 was the dose at which 50 / of the animals did not show this reaction in 15 sec.

FG 5111 chlorpromazine, and haloperidol showed activity in the analgesic tests at doses similar to the effective doses of morphine, but pethidine had a weaker effect (table 5)

Nalorphine, 12 mg/kg subcutaneously completely antagonized the analgesic effect of morphine (3 mg/kg) in the writhing test, but did not influence the analgesic action of 10 mg/kg FG 5111

Table 5

ED50 (mg/kg s. c.) in tests on analgesic activity

Compound	Writhing test	Hot plate method
FG 5111	[illegible]3 (1.5–3.4)	4.3 (2.8–6.6)
Chlorpromazine	2.3 (1.5–3 4)	3.5 (2.3–5 3)
Haloperidol	1.5 (1 0–2.3)	3 4 (2.[illegible] 5.3)
Morphine	1.2 (0.9–1.5)	7 6 (5.0–11.6)
Pethidine	1[illegible] (11–30)	[illegible]5 (20–32)

G *Anticonvulsant effect*

Maximal electroshock (MES) test.

A modification of the procedure described by Swinyard (1949) was employed. Mice were fixed by electrode clamps in the skin at the neck and the tail An electric current (100 V 20 mA, 0 2 sec.) induced maximal tonic seizures in 100 / of untreated controls. The test compound was administered s. c. or p o at different times before the electroshock was applied The ED50 was the dose protecting 50 / of the animals from tonic convulsions.

Pentetrazole (metrazol) antagonism test.

The test substance was injected subcutaneously into mice (6 animals/dose). Pentetrazole was injected subcutaneously 30 min. later 150 mg/kg. The ED50 was the dose protecting 50 / of the mice against convulsions within 30 min. after pentetrazole injection.

Strychnine antagonism test.

This test was carried out in the same way as the anti-pentetrazole test, except that strychnine nitrate, 1 mg/kg, was injected subcutaneously instead of pentetrazole

Injected subcutaneously into mice 30 min. before testing the anticonvulsant activity (el. shock, pentetrazole, strychnine) FG 5111 and haloperidol showed an anticonvulsant effect like that of phenytoin. Chlorpromazine had no anticonvulsant effect (table 6)

When administered orally to mice 2, 4 or 7 hours before testing anticonvulsant effect (el. shock) haloperidol had no effect and FG 5111 only a weak effect, compared with those of phenytoin and phenobarbital (table 7)

Table 6

ED50 (mg/kg s. c.) in tests on anticonvulsant effect.

Compound	Elektroshock	Metrazol	Strychnine
FG 5111	11 (8–15)	31) (25–37)	20 (16–25)
Chlorpromazine	>50	>50	>50
Haloperidol	6 (4–9)	15) (10–22)	20 (14–28)
Phenytoin	18 (14–23)	30) (24–37)	65 (54–78)
Phenobarbital	30 (23–39)	35 (28–44)	90 (72 112)

) Did not protect against clonic convulsions but only against the terminal tonic phase.

Table 7

Duration of anticonvulsant effect measured in the electroshock seizure test.

Compound	Electroshock ED50 mg/kg p. o.		
	2 h after administration	4 h after administration	7 h after administration
FG5111	75 (47–120)	75 (50–112)	>100
Haloperidol	>20	>20	> 20
Phenytoin	20 (13–31)	20 (13–32)	20 (14–30)
Phenobarbital	14 (9–21)	14 (9–21)	20 (13–31)

H *Tremorine antagonism.*

EVERETT *et al* (1956) reported that tremorine produces tremor and parasympathetic stimulation in laboratory animals. The test substance was administered to mice (6 mice/dose) before 20 mg tremorine/kg intraperitoneally The number of mice showing tremor or salivation within 30 min. after the injection of tremorine was recorded. The ED50 was the dose protecting 50 / of the mice against tremor or salivation.

Protection against tremorine-induced tremor in mice was shown by FG 5111 and haloperidol but they did not prevent the cholinergic effect of tremorine, measured by the profuse salivation (table 8) A similar antagonism against the effects produced by oxotremorine was shown by FG 5111 (B HOLMSTEDT personal communication 1964) The anti Parkinsonian agents atropine, scopolamine and benztropine completely inhibited all effects produced by tremorine.

Table 8

Anti-tremorine activity in mice.

Compound	Route	Interval to tremorine inj. min.	Protection against	
			Tremor ED50 mg/kg	Salivation ED50 mg/kg
FG 5111	s. c.	30	20 (13–32)	>50
–	p. o.	60	18 (12–37)	>50
Chlorpromazine	p. o.	60	>50	>50
Haloperidol	p. o.	60	10 (7 15)	~25
Atropine	p. o.	60	8 (5–12)	8 (6–11)
Scopolamine HBr	p o.	60	10 (6–16)	8 (5–12)
Benztropine	p. o	60	5 (3–7)	2 (1 3)

I *Amphetamine antagonism*

Rats were injected subcutaneously with D-amphetamine sulphate (3 mg/kg) About half an hour later the rats showed abnormal stereotyped behaviour as described by RANDRUP *et al* (1963) The duration of the typical stereotyped movements was about 2–3 hours. The test compound was given to 8 rats per dose

Apparently FG 5111 chlorpromazine and haloperidol antagonized amphetamine-induced abnormal behaviour (table 9) The effective doses of chlorpromazine produced sedation and ataxia, but the animals treated with effective doses of FG 5111 and haloperidol were nearly normal in appearance

Table 9

Inhibition f stereotyped amphetamine-induced activity in rats.

Compound	Route	Interval to amphetamine *inj. min.*	ED50 mg/kg
FG 5111	s. c.	0	1.3 (0.8–2.1)
–	p o.	60	15 (10–23)
Chlorpromazine	s. c.	0	1.3 (0.8–2.1)
–	p.	60	15 (9–24)
Haloperidol	s. c.	0	0.018 (0.012–0 028)
–	p. o.	60	0.20 (0.13–0 30)

K *Apomorphine antagonism.*

Rats injected intravenously with apomorphine show typical behaviour (JANSSEN *et al* 1960) This effect is antagonized by several tranquillizers and anti-emetics.

The test drug was administered subcutaneously (6 rats/dose) 1.25 mg/kg apomorphine by slow intravenous injection one hour later The rats were then placed in separate cages and observed for 20 min The ED50 was the dose at which 50 / of the animals did not lick or chew during the observation period.

The antagonistic action of FG 5111 against the special behaviour provoked by apomorphine in rats was relatively weak, the ED50 in mg/kg being 6.2 (5.3–7.3) compared with that of haloperidol, ED50 = 0.3 (0.2–0 4) but of the same order of magnitude as that of chlorpromazine, ED50 = 2.5 (1 7–3.8).

L. *IDPN antagonism.*

Administration of high doses of β-imino-dipropionitrile (IDPN) has been shown to provoke abnormal behaviour in mice, characterized by rapid circling movements, which persist for the rest of the animal's life (DELAY *et al* 1956). This abnormal activity may be suppressed by tranquillizers, e. g. chlorpromazine.

The mice were injected intraperitoneally with IDPN 1.5 g/kg daily for 4 days. When the circling activity had been established, they were divided into groups of 5 and the activity of the group was recorded by the photocell technique before and one hour after subcutaneous administration of the test drug (one group of 5 animals per dose level) The ED50 was the dose that reduced the IDPN-induced hyperactivity by 50 %.

Both FG 5111 and chlorpromazine, as well as haloperidol suppressed the hyperactivity caused by IDPN the ED50 in mg/kg being 3 6 (2.0–6.6) 3 0 (1 8–5 1) and 0.5 (0 3–1 1), respectively

M *Reserpine antagonism.*

The antagonism to reserpine-induced hypothermia was determined in mice. The test compounds were administered subcutaneously (6 animals per dose) before intraperitoneal injection of reserpine. The rectal temperature was measured thermoelectrically In each experiment 6 mice treated only with reserpine and 6 untreated mice served as controls The room temperature during the test was 19–20°

When administered 1 hour before the reserpine FG 5111 (0.1–10 mg/kg) showed no antagonistic effect.

Results of experiments involving injection of test drugs 4 hours before

Table 10

Effect on reserpine-induced hypothermia in mice.

Pretreatment mg/kg s. c.	Reserpine mg/kg i. p. 4 h after pre-treatment	Body temperature (°C ± S.E.M.)	
		At pretreatment	20 h after reserpine
FG 5111 – 4	5	36.9 ± 1.0	21 7 ± 1.9
FG 5111 –	5	37 4 ± 0.6	37 6 ± 0.3
FG 5111 – 1	5	36.9 ± 0.6	36 0 ± [illegible]
0	5	37 1 ± 0.5	19 7 ± 0.8
0	0	37.5 ± 0.6	36.9 ± 1.0
FG 5111 – 0.5	5	37.0 ± 0.8	[illegible]5 1 ± 1 7
FG 5111 – 0.25	5	37.3 ± 0.5	21 6 ± 0.8
0	5	37.5 ± 0 4	21 1 ± [illegible]
0	0	37.3 ± 1.0	37.9 ± 0.5
Chlorpromazine – 4	5	37 4 ± 0 5	21.5 ± 1.3
Chlorpromazine – 2	5	37.5 ± 0.5	20.2 ± 2.5
Chlorpromazine – 1	5	37.8 ± 0.2	20.8 ± 0.5
0	5	37 6 ± 0.4	20.1 ± 0.5
0	0	37.5 ± 0.2	36.9 ± 0 9
Haloperidol – 4	5	36.5 ± 0.8	19 7 ± 0.8
Haloperidol –	5	36.9 ± 0.5	21 0 ± 0 5
Haloperidol – 1	5	36.7 ± 0.6	20.7 ± 0.4
0	5	36.9 ± 0.3	20 4 ± 0.7
0	0	36.7 ± 0.5	36.9 ± 1.0
Haloperidol – 0.5	5	37 8 ± 0.3	21 3 ± 0 6
Haloperidol 0.25	5	37 5 ± 0.4	21.5 ± 1.9
Haloperidol 0.125	5	37.8 ± 0 5	20.1 ± 0.3
0	5	37 7 ± 1 0	21 7 ± 0.7
0	0	36.8 ± 0.6	36.9 ± 0.8

reserpine are shown in table 10 The hypothermia provoked by reserpine was inhibited by FG 5111 (1–2 mg/kg) which delayed the other symptoms (sedation, ptosis and diarrhoea) due to reserpine. In doses of 4 mg/kg and more FG 5111 did not antagonize symptoms due to reserpine Chlorpromazine (1–4 mg/kg) and haloperidol (0 125–4 mg/kg) administered 4 hours before reserpine exhibited no antagonistic effect.

N *Electroencephalographic studies*

The technique previously described by STERNER (1963) was used The EEG was recorded in unanaesthetized rabbits with chronically implanted electrodes.

The test substance was administered intravenously and the EEG was recorded continuously in connection with the injection and then at various increasing intervals The effects of different external stimuli were studied. In doses above 0.5–1 mg/kg FG 5111 chlorpromazine and haloperidol showed unambiguous EEG effects, which, however were different for FG 5111 and haloperidol on the one hand and chlorpromazine on the other The EEG effects of the two former compounds could be characterized by an initial phase of a continuous arousal pattern with a duration of 10 to 30 min., depending on the dose This phase gave place to slow high-voltage activity (with occasional short periods of fast activity) of 2 to 3 hours duration, during which a partial block of external stimuli could be obtained. This block was more complete for audiogenic stimuli than for stronger stimuli, e. g. mixed audiogenic and vibrational. Fig. 3 shows EEG records from one experiment with FG 5111

On the other hand chlorpromazine gave, soon after the injection, syn chronization of 2 to 3 hours duration, with at least partial block of arousal reactions on afferent stimulation (STERNER 1964)

Higher (toxic) doses of FG 5111 and haloperidol (6 mg/kg) gave a marked pattern of excitation in the EEG similar to that of an epileptic fit, whereas chlorpromazine, in doses over 6 mg/kg, produced continuous desynchronization of several hours duration and thereafter (with de creasing effect) the same EEG pattern as lower doses.

O *Effect on spinal reflexes*

The effect on two different spinal reflex pathways was determined in conscious denervated rabbits spinalized by sectioning the spinal cord at the tenth thoracic segment. The animals were allowed to recover for 24 hours. The patellar reflex (monosynaptic arc) was elicited mechanically The flexor reflex (polysynaptic arc) was provoked by electrical stimulation (10 V 1 msec, 0.3 cps) of the tibial nerve, which was cut peripherally The reflex responses were recorded kymographically The preparation was checked with mephenesin which, in doses of about 40 mg/kg intravenously should give a clear reduction of the flexor reflex without affecting the patellar reflex. The substance under investigation was injected intrave nously in increasing doses to determine the dose causing a complete block of the reflex response. Three experiments were done for each compound The ED50 was the dose causing 50 / inhibition of the reflex response

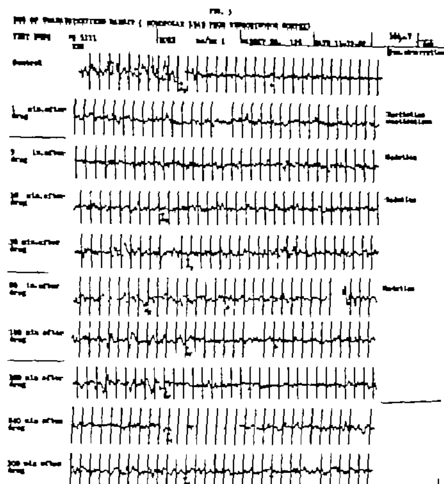


Fig. 3 Effect on EEG of unanaesthetized rabbit (monopolar lead from sensory-motor cortex). A_1 and A_2 = different types of audiogenic stimuli. AV = vibrational stim lus.

All the compounds FG 5111 chlorpromazine, haloperidol mephenesin and chlordiazepoxide in the doses tested had an inhibitory effect on the flexor reflex without affecting the patellar reflex (table 11) The effective flexor reflex inhibiting doses of FG 5111 chlorpromazine and haloperidol also caused depression of the blood pressure, so that the reflex inhibition might be secondary to the cardiovascular effect. In contrast, mephenesin and chlordiazepoxide showed no cardiovascular effects in doses producing reflex inhibition

Table II

Effect on flexor nd patellar reflexes in spinalized rabbits.

Compound	Acute average reduction dose	
	Flex r reflex ED50 (mg/kg) L	Patellar reflex ED50 (mg/kg) L
FG 5111	3.3	>10
Chlorpromazine	0.3	> 1
Haloperidol	0.3	> 1
Mephenesin	32 0	>80
Chlordiazepoxide	1.5	> 5

P *Effect on blood pressure*

Unanaesthetized rats.

Rats were placed in a special chamber that permitted plethyamographic measurement on the tail of the systolic blood pressure A modification of the technique described by BYRON & WILSON (1938) was used The rats were kept at a temperature of 28° for 1 hour before and during the experiments.

After subcutaneous injection of 1–20 mg/kg FG 5111 slight increases in blood pressure were seen, and 20–50 mg/kg caused more pronounced increases (25–70 mm Hg) of longer duration (20–140 min.).

Chlorpromazine (1–10 mg/kg) caused some depression of the blood pressure (10–40 mm Hg) lasting for 20–40 min.

Haloperidol (1–5 mg subcutaneously) decreased blood pressure by 10–25 mm Hg for about 30 min. and 5–10 mg/kg increased blood pressure by 40–50 mm Hg for 90–180 min

Figure 4 shows some effects on blood pressure recorded for the three compounds.

FG 5111 exerted no adrenaline antagonism in doses from 1 to 50 mg/kg subcutaneously Chlorpromazine (0.5–2.5 mg/kg) reduced, suppressed and even reversed the rise of blood pressure produced by adrenaline Haloperidol (1–10 mg/kg) had no anti-adrenaline effect. Fig. 5 shows the effects on blood pressure of 10 mg FG 5111/kg, 2 mg chlorpromazine/kg, and 2 mg haloperidol/kg subcutaneously 30 min. before 0.5 mg adrenaline/kg intraperitoneally

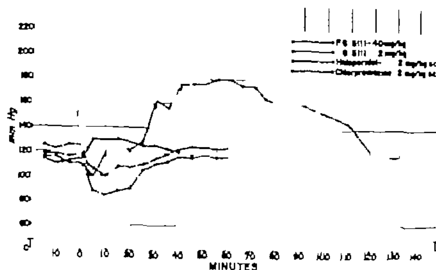


Fig. 4. Effect on blood pressure in unanaesthetized rats.

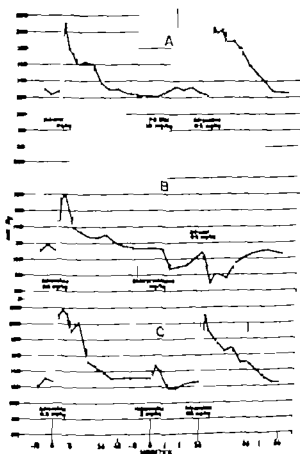


Fig. 5. Antiadrenaline activity measured by blood pressure effect in unanaesthetized rats.

Anaesthetized cats.

Experiments on cats were performed under surgical anaesthesia (ether + chloralose). The left common carotid artery and the trachea were connected to Statham pressure transducers and a Honeywell Visicorder for the recording of blood pressure and respiration, respectively. The right common carotid artery was prepared for occlusion and the right vagus nerve for stimulation. Test substances were administered intravenously (through the cannulated left femoral vein) in increasing doses. The effects on the blood pressure were recorded and so were the alterations in standard responses produced by each of the six procedures: 1. Adrenaline (5 γ/kg), 2. noradrenaline (5 γ/kg), 3. isoprenaline (4 γ/kg), 4. acetylcholine (5 γ/kg), 5. occlusion of the carotis for 45 seconds, and 6. electrical stimulation of the vagus for 15 seconds (3 V, 10 msec, 0,3 cps).

In doses of 100–1000 μg/kg FG 5111 gave a brief fall in blood pressure (25–35 mm Hg) with at higher doses a subsequent slight secondary fall of 15–30 minutes duration. The carotid occlusion reflex was depressed and the rise of blood pressure after injection of adrenaline or noradrenaline slightly reduced (fig. 6).

Chlorpromazine in doses of 20–800 μg/kg produced, after a latent period of 3–6 min., a long lasting (several hours) fall of blood pressure (10–30 mm Hg), sometimes preceded by a short lasting fall. The effect of chlorpromazine on carotid occlusion and on adrenaline and noradrenaline injections was somewhat stronger and longer lasting than that of FG 5111 (fig. 6). Haloperidol in doses of 100–1000 μg/kg exerted approximately the same effects as FG 5111 (fig. 6).

Q. *Local anaesthetic effect*

Surface anaesthesia in rabbits.

A 1 % solution of lidocaine was instilled into one eye of a rabbit and a 1 % solution of the test substance into the other eye. The corneal reflex was tested at intervals of 2 min. by touching the cornea with a thin glass rod, and the time was recorded during which the reflex was blocked. If the test compound showed a local anaesthetic action, the test was repeated with lower concentrations of both substances. The value of the local anaesthetic effect was calculated as a relative activity (lidocaine = 1).

Infiltration anaesthesia in guinea pigs.

The method of BULBRING & WAJDA (1945) was used.

In the guinea-pig infiltration test FG 5111 showed a clear local anaesthetic action, which could not be detected in the tests on surface anaesthesia in rabbits (table 12).

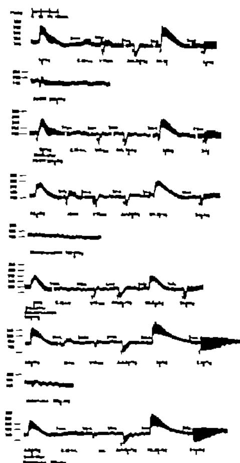


Fig. 6. Effect on blood pressure in anaesthetized cats.
A = adrenaline. C = carotid occlusion. V = vagus stimulation.
Ach = acetylcholine. NA = noradrenaline. I = isoprenaline.

Table 12

Local anaesthetic activity of FG5111 chlorpromazine and haloperidol compared with that of lidocaine.

Compound	Surface anaesthesia (relative potency)	Infiltration anaesthesia (relative potency)
Lidocaine	1	1
FG 5111	0	0.9
Chlorpromazine	3	1
Haloperidol	4	1.8

Table 13

Antagonism of stimulant standard substances on isolated guinea-pig ileum (ID50 μg/ml bath).

Compound	Acetylcholine	Histamine	Serotonin	Nicotine
FG 5111	20–200	20	20	20
Chlorpromazin	0.2 2	0.02–0.2	0.0 –0.2	0.02–2
Haloperidol	2–20	1	1	1 2
Atropine	0.01–0.02			
Diphenhydramine		0.01–0.02		
Hexamethonium				2 20

R. *Effect on isolated rat diaphragm*

The neuromuscular blocking action of the test substance was investigated on isolated rat diaphragm – phrenic nerve preparations. The nerve was stimulated electrically (0.2–0.8 V 10 msec, 0.33 cps) and the contractions of the diaphragm were recorded kymographically by means of an isotonic lever Direct stimulation (2–4 V 10 msec, 0.33 cps) of the muscle was used to ascertain the site of action (nerve incl. neuromuscular junction or muscle) of the substance.

In this preparation FG 5111 chlorpromazine and haloperidol blocked muscle contraction, but relatively high doses were required for a 100 / block (2.1 2.5 and 0 8 mg/bath, respectively) compared with that of curare (100 μg/bath). The local anaesthetic lidocaine had a blocking action to about the same degree as FG 5111

Only curare was antagonized by neostigmine. Direct stimulation of the muscle was blocked by about the same dose of FG 5111 as indirect stimulation, indicating that the site of action was not the neuro-muscular junction.

S *Effect on isolated guinea-pig ileum*

Isolated guinea-pig ileum was used for studying direct stimulant activities on smooth muscle and antagonisms to acetylcholine, histamine, serotonin and nicotine Atropine, diphenhydramine and hexamethonium were used as reference substances. The ID50 was the dose causing a 50 / inhibition of the response to the standard substances.

No direct stimulation of smooth muscle by FG 5111 was found, and the antagonism of the standard stimulants, acetylcholine, histamine, serotonin and nicotine, was weak (table 13)

Table 14

Acute toxicity in mice and rats expressed as LD50 i mg/kg.

Compound	Route	Mice	Rats
FG 5111	i. v	35 (25–55)	40 (27–58)
–	s. c.	230 (177 300)	220 (154–314)
–	p. o	230 (180–294)	330 (216–505)
Chlorpromazine	i. v	37 (25–55)	30 (21–43)
–	s. c.	250 (205–310)	300 (185–485)
–	p o.	290 (220–382)	370 (230–600)
H loperidol	i.	30 (21–4)	20 (14–28)
–	s. c.	60 (48–75)	75 (52 108)
–	p.	75 (59–95)	>75

T *Acute toxicity*

The test compound was administered to groups of mice and rats in increasing doses. The animals were observed for 48 hours, and the lethality after that time was used for determining the LD50 value

Table 14 shows the LD50 values for FG 5111 chlorpromazine and haloperidol. After administration of FG 5111 and chlorpromazine in doses approaching the LD50, the animals showed hypothermia and loss of righting and pain reflexes. The duration of the symptoms was 3–8 hours with FG 5111 and up to 48 hours or more with chlorpromazine The animals died after lethal doses of FG 5111 2–18 hours after subcutaneous or oral administration and within 2 hours after intravenous injection. With lethal doses of chlorpromazine the animals died in 18–48 hours by subcutaneous or oral administration and in 1–48 hours after intravenous injection.

The animals treated subcutaneously with haloperidol (30 mg/kg and over) showed clonic convulsions of 1–3 hours duration some of the animals survived the convulsions and then showed symptoms similar to those of animals treated with FG 5111 or chlorpromazine. After intra venous injection of haloperidol in doses approaching LD50, clonic convulsions of shorter duration were observed. With haloperidol the duration of the tranquillizing symptoms and the time of death after lethal doses were about the same as with FG 5111

U *Subacute toxicity in rats*

For a period of 15 weeks FG 5111 was administered orally to 20 young rats weighing about 100 g (10 males and 10 females) The drug was given daily in a dose of 30 mg/kg on Monday to Thursday and twice on Friday A corresponding group of rats receiving physiological saline served as controls. The food consumption and growth were recorded throughout

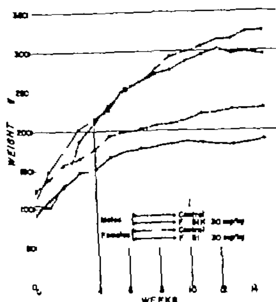


Fig. 7 Growth curves of FG 5111-treated and saline-treated (control) rats.

the test, and every 5'th week the blood picture (Hb-values, red and white blood cell counts, differential counts) was checked. At the end of 5 and 10 weeks four rats and after completion of the test the remaining rats were killed, and the weights and macroscopic appearances of various organs (heart, spleen, liver kidneys, adrenals, thyroid and pituitary glands) were examined. The liver kidneys, adrenals, thyroid, pituitary and bone marrow were studied histologically also [1])

As shown in figure 7 the growth of the animals receiving FG 5111 was somewhat less than that of the controls, probably owing to the sedative action of the substance and a secondary appetite-depressing effect. The blood picture or the weight and appearance of organs from FG 5111 treated animals did not show any differences from those of untreated animals. Histological examination revealed no pathological changes attributable to the treatment.

Discussion

From a number of GABA derivatives exerting psychotropic effects FG 5111 was selected, and its psychopharmacological "activity profile" was determined in comparison with those of two well-known psychotherapeutic drugs, chlorpromazine and haloperidol

[1]) Performed by K. Schourup M.D chief pathologist, Copenhagen County Hospital, Glostrup.

Fig. 8 Activity profiles" f FG 5111 chlorpromazine and haloperidol. Along the vertical axis are the log ED50's in various tests described in the text. The upper and lower edge of each rectangle show the 95 % confidence limits. The arrows indicate that no effect was seen in doses up to those mentioned.

1) Did not protect against clonic convulsions.
2) Test substances administered orally
3) No protection from cholinergic effects.

Fig. 8 shows similarities and differences between the compounds studied in tests in which the ED50 was determined. The compound FG 5111 had an effect on spontaneous activity and motor co-ordination at the same dose levels as chlorpromazine. Unlike chlorpromazine, FG 5111 showed some anticonvulsant effect, but this was weaker than that of phenytoin or phenobarbital. It inhibited tremorine-induced tremor but did not protect against the cholinergic effects of tremorine. Haloperidol was also effective in the anticonvulsant tests and the anti-tremorine test, but less so than FG5111

In tests generally used to determine specific psychosedative activity (depression of conditioned reflex and amphetamine antagonism) all three compounds were effective haloperidol showed remarkably strong antagonism to amphetamine. The fact that the structurally simple compound FG 5111 exerted an activity in these tests may indicate its possible clinical value in various mental disorders.

The three compounds were active in tests used to measure analgesic activity but at dosages affecting spontaneous activity and motor coordination the analgesic effect may thus be unspecific.

The observed antireserpine effect of FG 5111 is puzzling and not due to MAO inhibition (SQUIRES, personal communication 1964)

The compound had relatively weak autonomic effects, and it is for future clinical experiments to show whether it consequently gives rise to fewer side effects than other neuroleptics.

Summary

This describes a comparative pharmacological and toxicological study of FG 5111 (γ-(4-methylpiperidino)-p-fluorobutyrophenone), chlorpromazine and haloperidol.

The compound FG 5111 appears to possess a neuroleptic activity similar to those of chlorpromazine and haloperidol Like the latter substance, FG 5111 has a weak anticonvulsant and antitremorine effect as well as a biphasic action on the cortical EEG of rabbits, with an initial desynchronization and subsequent synchronization. In contrast to the reference substances, FG 5111 shows some antireserpine properties.

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Histochemical Demonstration of Adrenergic Nerves in Cortex Pia of Rabbit

By

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There are several reasons for believing that adrenergic mechanisms are operative in the cerebral cortex. Thus, catecholamines have been detected chemically although in fairly small amounts in the cortex compared with those in certain "deeper" structures of the cerebral hemispheres (VOGT 1954 CARLSSON 1959 KUNTZMAN *et al* 1961 HATTORI 1964) The specific enzymes responsible for synthesis and metabolism of monoamines are present in cortical tissue (*cf* KUNTZMAN *et al* 1961 WEINER 1961 MCCAMAN & APRISON 1964 MCCAMAN *et al* 1965) Further cortical slices have been shown to take up and concentrate catecholamines, especially noradrenaline, against a considerable gradient (DENGLER, SPIEGEL & TITUS 1961 TITUS & DENGLER 1962 ROSS & RENYI 1964) This is a characteristic feature of adrenergic neurons both in the central nervous system (FUXE & HILLARP 1964 FUXE & OWMAN 1965) and in peripheral tissues (HAMBERGER *et al* 1964 HILLARP & MALMFORS 1964) Finally it has been demonstrated that single cortical neurons readily respond to the application of different monoamines (KRNJEVIĆ & PHILLIS 1963)

In order to understand adrenergic mechanisms it is, however necessary to localize the monoamines at the cellular level. Recently FALCK & HILLARP devised a method for the demonstration of certain biogenic monoamines by fluorescence microscopy (FALCK 1962 FALCK *et al* 1962 CORRODI & HILLARP 1963 & 1964) sensitive enough to make visible *e.g* intraneuronally located noradrenaline (FALCK & TORP 1962) Our investigation was concerned with direct histochemical localization of adrenergic nerves, both in cortical tissue and around the pial vessels as well as in the radial arteries penetrating into the rabbit cortex.

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Material and Methods

The tissues to be analyzed histochemically by the method of FALCK (1962) and FALCK & OWMAN (1965a) were obtained from rabbits of both sexes. Six normal animals were studied. Reserpine (Serpasil, Ciba) was administered in a dose of 5 mg/kg subcutaneously to four animals, which were killed after 24 hours. A monoamine oxidase inhibitor nialamide (Niamid, Pfizer) – was injected intraperitoneally (150 mg/kg) into three animals, which were killed 6 hours later. In another series, four animals were first subjected to bilateral cervical sympathectomy by *removing the superior cervical ganglia under mebumal* (nembutal) anaesthesia, and then on the 4th postoperative day injected with 200 mg/kg of nialamide, which was repeated after 16 hours; the animals were killed 5 hours after the last injection. Bilateral cervical sympathectomy alone was done on five animals, which were *killed 4 days later. Finally, three animals were given 200 mg/kg of α-methylmetatyrosine* by the intravenous route and killed 24 hours later.

The animals were killed by intravenous air (from some the tissues to be studied were removed under urethane anaesthesia), and pieces of the cortex (two to eight per animal), including the pia mater, measuring about 4 × 4 × 3 mm were dissected out. As control organs for the histochemical reaction and the pharmacological treatments, pieces of the heart ventricles were also included. The specimens were treated in accordance with the description given by FALCK & OWMAN (1965a). This includes formaldehyde condensation of the monoamines, whereby the catecholamines and 5-hydroxytryptamine form intensely fluorescent products emitting a green and a yellow light, respectively, under the conditions of fluorescence microscopy used.

A simple technique was used for pial tissue. Four animals were killed as described above, and under a dissecting microscope small pieces of pia measuring about 4 × 4 mm, were stripped off from the underlying cortex and stretched on a slide, which was dried for one hour at room temperature under diminished pressure. After the usual treatment in formaldehyde gas, the preparations were analysed directly in the fluorescence microscope.

Results

In the cerebral cortex of the normal rabbit a distinct fluorescence of green colour, characteristic for catecholamines, developed only in delicate varicose fibres. The fluorescence was not of a very high intensity although it stood out clearly against the essentially dark background of the brain tissue. Only few nerve fibres of fairly large calibre emitted a more intense light. All the fibres (fig. 1) had the same characteristic appearance as the adrenergic nerve *terminals elsewhere* in the central nervous system (*cf.* CARLSSON *et al.* 1962 & 1964) or in peripheral tissues (NORBERG & HAMBERGER 1964, FALCK & OWMAN 1965b). No difference in the characteristics of the fluorescence was observed whether the preparations were dissected out *in vivo* or immediately *post mortem* (*cf.* CSILLIK & ERULKAR 1964). The fluorescence intensity was not enhanced in preparations treated in formaldehyde gas for three hours as against one hour. Thus, a primary, not a secondary, catecholamine was in all probability present (see FALCK & OWMAN 1965a).

In the outer layers of the cortex, the fluorescent nerves were most







Fig. 1 Terminal varicose parts of adrenergic axons running in the neocortex of normal rabbit

(a) Long varicose nerve, measuring about 0.6 mm and running in fairly straight course. 180.

(b) Axon running contiguous to three non-fluorescent cell bodies (arrows) The rounded black areas represent the cell nuclei. × 310.

( ) Varicose fibre, which ends on a non-fluorescent cell body (upper part of the figure). 310.

abundant and ran mainly parallel to the cortical surface (fig. 2) They were usually short, not exceeding 0 1 mm, but sometimes the fibres were fairly long (fig. 1a) Some of the fibres ran superficially lying in close proximity to the pial vessels. In the deeper layers the nerves traversed the cortical nervous tissue more irregularly in all directions, to create a three dimensional rather scanty meshwork of fibres (fig. 3) The course of the nerves was largely straight, but here and there bending to pass contiguous to non-fluorescent nerve cell bodies (fig. 1b) Sometimes the fibres seemed to end on such a cell body or on the most proximal part of its axonal



Fig. 2 Abundance of fluorescent nerves, many of which ru parallel to the surface (upper part of the figure) in the outer layer f the neocortex. In th pia at the surface a few autofluorescent cells are seen. Sympathectomized rabbit treated with nialamide. ×180



Fig. 3 Deeper layers f neocortex Fluorescent, varicose nerves running in all directions between non-fluorescent nerve cells, whose uclei re seen as black reas Sympathectomized rabbit treated with ialamide. 200

process, after having run on its surface (fig. 1c) The nerve fibres often crossed the course of radial blood vessels to the brain, but in no instance were the fibres seen to cross the perivascular space to run along the vessels.

Fluorescent granular material was often seen in the cell bodies The fluorescence was present even in preparations not treated with formaldehyde and thus represents autofluorescent granules. Moreover this material emitted light in the brown yellow range, and this in contrast to the fluorophores of the biogenic monoamines, did not fade in ultraviolet light finally it was not affected by reserpine (see below)

In the pia mater varicose nerves having the same morphology and calibre as those in the cortex, but emitting a fairly intense green light, ran along the blood vessels often in small bundles of two or three fibres, with frequent intercommunications between the bundles. This gave rise to a wide-meshed plexus superimposed upon the media. The innervation was by no means as rich as that seen in certain peripheral vascular beds (FALCK 1962 NORBERG & HAMBERGER 1964 FALCK & OWMAN 1965b) The pial veins were enclosed by even fewer fibres, arranged in a similar manner as in the arterial plexuses. The fibre system around the radiating vessels of the brain substance showed the same construction. These nerves could often be followed from the pial vascular nerve plexuses on the cortical surface, where they took a new and essentially perpendicular direction to continue into the brain tissue, where they ran along the radiating vessels.

Sometimes the pial vascular nerves seemed to run directly into the outer layers of the cortex. When such nerves were followed in serial sections, it became obvious that they were sometimes related to the radial vessels. In other instances, however the nerves were seen to leave the arteries at a conspicuously acute angle and continued in this direction within the cortex without relation to the vessels. Often the course of a nerve fibre was interrupted for a short distance where it passed through a narrow space between the pial membrane and cortex. This loss of continuity was evidently an artefact occuring during the quenching or sectioning of the preparation. A further feature, especially evident in the stretch-preparations of whole pial membranes, was that the vascular nerve plexuses of adjacent vessels showed interconnections in the pia tissue by single fibres or small bundles of varicose nerves. The pia contained several cells filled with granular material showing an orange autofluorescence (fig. 2), but they did not interfere with the intraneuronal amine localization.

After injection of the monoamine oxidase inhibitor nialamide, the green fluorescence of the cortical nerve fibres became somewhat more intense. Nerve fibres obviously without any notable fluorescence under normal conditions now exhibited a more or less intense green light. The

general arrangement did not differ from that observed in the untreated animal. No increase occurred in the fluorescence of the vascular nerves. No change was noted in the autofluorescent cells, as was to be expected. Nor did any cell bodies with specific fluorescence show up after the nialamide administration.

Reserpine treatment caused a complete disappearance of the fluorescence in all types of nerves of the cortex preparations, as well as in the nerve fibres of the control tissue (heart). No change occurred in the autofluorescent material.

Bilateral cervical sympathectomy along with nialamide administration (fig. 2 & 3) caused a total loss of fluorescence from all vascular nerves. The same results as those with nialamide alone were shown by the fluorescence intensity and the number of fluorescent cortical nerve fibres.

After bilateral cervical sympathectomy only the fluorescence in all vascular nerves was abolished. No changes could be seen in the number or fluorescence intensity of the intracortical axons.

α-Methylmetatyrosine caused a severe depletion of monoamines in cortical and vascular nerves. In some of the animals the cortex showed only few nerve fibres with extremely faint fluorescence. The fluorescence in the vascular nerves was somewhat less reduced, but remained in a few locations only, particularly where the fibres ran close together in bundles. A depletion had also occurred in the adrenergic nerves of the heart: the number of fibres was seriously reduced, and the fluorescence of the remaining ones was less intense than in the normal animal.

Discussion

In preparations from the cerebral cortex and pia mater of the rabbit a system of fine varicose fibres having the same appearance as the terminal parts of adrenergic axons in peripheral organs (see FALCK 1962, NORBERG & HAMBERGER 1964, FALCK and OWMAN 1965b) have been demonstrated. The colour of the emitted light and the reaction conditions under which it developed suggest the occurrence of a primary catecholamine in the nerves (FALCK & OWMAN 1965a). Similar nerves have also been demonstrated in neocortical tissue from rat (CARLSSON, FALCK & HILLARP 1962, FUXE 1965a & b) and man (FALCK & OWMAN, unpublished observations).

Reserpine and α-methylmetatyrosine, when given in doses causing disappearance of catecholamines from nervous tissues (CARLSSON *et al.* 1957, CARLSSON & LINDQVIST 1962), more or less abolished the fluorescence in the cortex-pia nerves. Inhibition of the monoamine oxidase that takes part in the inactivation of the monoamines (SHORE *et al.* 1957) led to an increase in the intensity of the fluorescence in the nerve fibres and the

number of fluorescent nerves. This is in agreement with the fact that monoamine oxidase inhibitors cause a considerable increase in the monoamine levels of the brain (CARLSSON, LINDQVIST & MAGNUSSON 1960). Thus, the results of the pharmacological treatments described furnish further strong evidence for the assumption that the nerves contain a monoamine.

The fluorescence reaction does not directly permit any distinction between noradrenaline and dopamine. α-Methylmetatyrosine has been found to cause a fairly long-lasting depletion of noradrenaline in nervous tissue, whereas the dopamine levels soon return to normal (CARLSSON & LINDQVIST 1962). The fact that the fluorescence of the cortical nerves was found to be strongly decreased even 24 hours after the administration indicates that the primary catecholamine involved is – at any rate mainly – noradrenaline.

The fluorescent nerves enclosed the pial arteries and also formed intercommunications between adjacent vascular nerve plexuses in the pia mater. A small number of fluorescent fibres coursed along the radiating arteries of the cortex. All vascular nerves are postganglionic axons running in the superior cervical ganglia, as demonstrated by the complete abolition of the fluorescence on removal of these ganglia. This loss of fluorescence in the distal stump of transected sympathetic fibres has been shown to precede the structural disappearance of the axons (FALCK 1962, HÅKANSON & OWMAN 1965).

Green-fluorescent nerves were also present in large amounts in the cerebral cortex, especially in the superficial layers. The cortical axons often ran, and sometimes ended, in close contact with non-fluorescent cell bodies. The contact did not take the form of a characteristic basket formation of fibres as seen in, for example, certain areas in the hypothalamus or in the spinal cord (CARLSSON *et al.* 1962 & 1964). This, however, by no means excludes the possibility that the nerve fibres in question form a synaptic contact with the cell bodies.

Sympathectomy had no apparent effect upon the cortical fibres, which thus seem, at least essentially, to originate from intracerebral cell bodies whose localization is as yet unknown. The existence of adrenergic cell bodies at different sites of the central nervous system is well known (CARLSSON, FALCK & HILLARP 1962, DAHLSTRÖM & FUXE 1964, FUXE & OWMAN 1965). In our investigation it was not possible to determine whether or not any cortical fibres were lost after sympathectomy, owing to the scattered arrangement of the nerves. Such nerve fibres might then have originated from the sympathetic fibres around the vessels in the pia mater. In fact, a small number of axons arising from the sympathetic plexus around the pial vessels were seen to issue into the cortex without

having any further obvious connection with blood vessels in the cortical substance.

It is known that increased functional activity of the cerebral cortex is accompanied by increased cortical blood flow and that the pial arterial system seems to play an important role in this regulatory mechanism (cf. SCHMIDT 1960, MCHEDLISHVILI 1964). Recent results indicate that the "nutritive" dilatation of the pial arteries is under direct nervous control by the cortex (MCHEDLISHVILI & DEVDARIANI 1964, MCHEDLISHVILI & NIKOLAISHVILI 1964). The possibility of an adrenergic mechanism being involved in the physiological regulation of the pial arterial system (OWMAN, FALCK & MCHEDLISHVILI 1965) is now under investigation.

Summary

With a fluorescence microscopic method for the cellular localization of certain monoamines, a fairly rich distribution of noradrenergic nerves has been demonstrated in the cerebral cortex of the rabbit. The pial vessels and the radial arteries receive a scarce innervation of postganglionic noradrenergic fibres arising in the superior cervical ganglia. A small number of the pial nerves probably issue into the cortex, although the bulk of cortical noradrenergic axons originate from intracerebral cell bodies of as yet unknown location.

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The Effect of Atropine, Histamine Liberators and Antihistamines on the Absorption of $DF^{32}P$ by Mice, Rats and Guinea Pigs

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(Received May 3 1965)

In a previous paper (RAMACHANDRAN & ÅGREN 1963a) it was shown that the absorption rate of radioactive diisopropylphosphorofluoridate ($DF^{32}P$) by rat tissues was considerably reduced if the organphosphate was mixed with atropine before injection. As this observation may have some practical significance, especially for the mode of administration of oxime antidotes against organphosphate poisoning (RAMACHANDRAN & ÅGREN 1963b) further studies have been undertaken to obtain information about the mechanism of this phenomenon. One of the causes for the reduced absorption rate may be the release of histamine at the site of injection, the so-called phenomenon of "self-depression" (SCHOU 1958) However self-depression has so far been demonstrated only in the rat (SCHOU 1961). In our studies it is now shown that atropine retards the absorption of $DF^{32}P$ in the mouse also Some results are presented on the effect of other atropine-like compounds on the absorption rate, as also on the effects of the powerful histamine liberator Compound 48/80 and the anti histaminic drug mepyramine on the atropine induced depression.

Materials and Methods

The Radiochemical Centre, Amersham, supplied the $DF^{32}P$ It had a specific activity of about 300 mC/g A stock solution was prepared in propylene glycol to contain approximately 1 mg of DFP in 1 ml and was preserved at −16 Quantities of the stock solution were weighed and diluted ten-fold with physiological saline just before the experiments.

The atropine sulphate used was of pharmacopoeial quality obtained locally The other compounds used and their sources were Mepyramine maleate (antihisan ®, May & Baker) Histamine dihydrochloride (Merck, Darmstadt) Scopolamine hydrobromide (Sandoz, Basel) Homatropine hydrobromide (Boehringer Ingelheim am Rhein) Cortisone acetate (cortone ®, Merck Sharpe & Dohme) Compound 48/80 (Wellcome Research Laboratories, Beckenham, Kent) Oxyphenonium bromide (antrenyl ® Ciba) Hyoscyamine sulphate (Nutritional Biochemicals, Cleveland, Ohio). The amounts of the bases mentioned in the text refer to their salts.

Male albino mice weighing from 25 to 30 g were used. The rats used were of the Sprague-Dawley strain and weighed from 200–250 g. The injection volume of $DF^{32}P$ was invariably 0.1 ml to mice. This volume contained approximately 0.01 mg of $DF^{32}P$ and gave countings of 0.2 to 0.8 × 10^6 cpm, depending on the extent of decay of ^{32}P The volume injected into rats and guinea pigs was 1 ml. When atropine or other adjuvants were to be incorporated, the test substances were dissolved in the $DF^{32}P$ solution, adjustments being made in the NaCl content so as to give isotonicity

The experimental procedure consisted in injecting an animal subcutaneously in the back and killing it after the necessary time interval. Mice and rats were stunned and decapitated guinea pigs were killed under ether anaesthesia. The liver kidneys and lungs were homogenized in saline in a Potter Elvehjem type of homogenizer and 1 ml portions of the uniform suspensions were pipetted into glass cups and dried at 110° after adding a drop of 5 N-NaOH. The brain tissue was prepared by dissolving it in hot 1 N-NaOH Radioactivity determinations were carried out either in a Tracerlab SC 18 Superscaler or in a Robot Scaling Equipment (LKB-Produkter Stockholm) fitted with a TGC 2 end-window Geiger tube.

Groups of 5 mice were usually employed for each determination, but the analyses were carried out separately for each animal. Variations in the volume of $DF^{32}P$ solution injected are estimated at 0.005 ml, introducing an error of ±5% from this source.

Results

Atropine-induced retardation of absorption in mice

When $DF^{32}P$ is mixed with atropine and injected subcutaneously into mice, the rate of absorption is considerably decreased. Fig. 1 shows the uptake of $DF^{32}P$ in the liver the kidneys, the lungs and the brain, with and without atropine, at various intervals after the injection The values are for the activity in the whole organs as a percentage of the total amount of activity injected. The average weights of the organs were liver 1 70 ± 0.26 g, kidneys 0 48 ± 0 12 g, lungs 0.22 ± 0.04 g and brain 0.38 ± 0.05 g (n = 200) The lung tissue was sometimes highly contaminated with blood It is seen that there is a considerably reduced uptake at the 15 min. interval by all the organs. The difference between the control and the experimental values decreases progressively with time

The extent of the retarding effect depends on the concentration of atropine used Table 1 gives the amounts of $DF^{32}P$ incorporated in the organs at various dose levels of atropine and at 3 selected times of killing, viz., 15 min., 30 min and 6 hrs The results are expressed as percentages

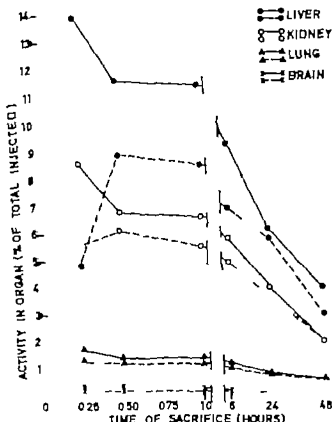


Fig. 1 The rate of incorporation of DF³²P in mouse organs. Each point represents the average value fo 5 mice. Groups of 5 mice were injected with DF³²P or a mixture of DF³²P and tropine at 30 mg/kg in volume of 0 1 ml and counting 232 × 10⁴ cpm due to ³²P The animals were killed after the required interval of time and the radioactivity in the organs was measured as described under methods. Continuous lines represent the controls and the broken lines those of experiments with atropine.

of the values in control experiments carried out simultaneously When more than 6 animals were used in a series, the results of 2 or more sets have been pooled. For liver there was a pronounced reduction in the uptake of DF³²P at the 15 min. interval even at low concentrations of atropine. At the 6 hr period there was a significant difference between the ³²P contents of atropine treated and control mice In the kidney the amount incorporated at the 15 mm interval was less in the presence of atropine, but the difference between the experimental and the control values is not of the same magnitude as that for the liver at the 30 min and 6 hr intervals. At low concentrations of atropine, viz., 10 and 5 mg/kg

Table 1

Incorporation of $DF^{32}P$ in mouse organs in the *presence* of various doses of atropine.

Organ	Atropine mg/kg	15 min. Activity as % of control	15 min. S.D. ±	30 min. Activity as % of control	30 min. S.D. ±	6 hrs. Activity as % of control	6 hrs. S.D. ±
Liver	Nil	100 (20)	4.9	100 (45)	2.7	100 (15)	2.9
	50	43 (30)	4.9	62 (11)	5.9	78 (10)	7.7
	40	59 (8)	4.6	69 (6)	7.2	87 (5)	12.0
	30	46 (10)	3.7	69 (10)	6.3	75 (5)	3.9
	20	58 (5)	6.1	64 (11)	6.9	77 (10)	4.6
	10	70 (10)	8.0	72 (11)	8.8	87 (10)	6.8
	5	75 (10)	6.9	88 (5)	5.9	90 (10)	5.1
Kidney	Nil	100 (20)	2.8	100 (40)	2.5	100 (15)	3.3
	50	63 (15)	5.1	89 (11)	4.7	90 (10)	3.6
	40	57 (5)	6.8	81 (6)	7.2	90 (5)	3.6
	30	59 (10)	7.4	93 (10)	3.6	83 (5)	6.2
	20	82 (10)	7.1	93 (11)	4.6	105 §(10)	5.3
	10	88 (10)	6.3	94 (10)	4.4	102 §(10)	5.1
	5	90 (10)	7.0	102 §(10)	6.2	104 §(4)	5.6
Lungs	Nil	100 (20)	4.3	100 (35)	2.2	100 (14)	2.5
	50	89 (15)	3.2	101 §(5)	8.4	106 §(5)	7.0
	40	82 (10)	5.7	98 §(6)	2.7	99 §(5)	3.8
	30	85 (10)	6.0	89 (10)	5.9	88 (5)	6.9
	20	87 (10)	6.5	103 §(11)	4.6	100 (5)	11.2
	10	90 (10)	4.6	91 (4)	7.7	103 §(10)	2.9
	5	97 §(10)	3.7	83 (5)	8.3	100 (10)	6.2
Brain	Nil	100 (20)	4.6	100 (28)	3.7	100 (15)	5.2
	50	51 (15)	7.3	76 (11)	8.9	92 §(10)	10.4
	40	67 (5)	13.7	87 (6)	6.8	87 (5)	4.8
	30	70 (5)	11.1	90 (5)	14.1	87 (9)	3.6
	20	66 (5)	7.6	69 (5)	7.3	105 §(9)	6.9
	10	90 (10)	5.5	90 (9)	2.6	106 §(9)	10.2
	5	93 §(10)	5.1	106 §(5)	13.5	102 §(10)	2.8

Groups of 4 to 6 mice were injected subcutaneously 0.1 ml each of $DF^{32}P$ solution, either alone or containing the required amount of atropine. The animals were killed after the required interval, and the organs were analysed individually as described under methods. The values are expressed as percentages of those for the control groups. The figures in brackets are the numbers of animals. The results of 2 or more groups have been combined when more than 6 animals are indicated. To conserve space, values of t and P are omitted but all figures except those marked § differ significantly (P = 0.05) from the control values.

and at longer intervals, the differences were not statistically significant.

The uptake in the lung tissue followed a different course from that in the other organs, in that there was considerable incorporation even at the 15 min. interval in the presence of high doses of atropine. The gap between the control and the experimental became insignificant at the 30 min. and 6 hr intervals. Erratic values were sometimes obtained owing to the presence of high amounts of blood in the lung tissue. The uptake by the brain was slow in the presence of atropine, the effect being comparable at the 15 and 30 min. intervals with that on liver. At the 6 hr period however the differences were hardly significant. In general the retarding effect of atropine in mice was much less than in rats, as reported earlier (RAMACHANDRAN & ÅGREN 1963a & b) and below (see table 3)

The effect of other substances on the rate of absorption in mice

Below the numbers in brackets correspond to the serial numbers of experiments reported in table 2. Hyoscine (scopolamine) (2), hyoscyamine (3), homatropine (4), oxyphenonium (5) and morphine (21) when administered at similar molar concentrations, depress the rate of absorption of $DF^{32}P$. Oxyphenonium was the most effective, as also found by SUND & SCHOU (1964) on rat muscles. Hyoscine was the least active. From the degree of inhibition obtained with hyoscyamine, it is not possible to conclude whether or not the optically active component of atropine was alone responsible for the depressing effect.

The extent of retardation by atropine was not affected to an appreciable degree by the systemic administration of mepyramine 45 min. before the injection of atropine $DF^{32}P$ mixture (6–9). Mepyramine is known to abolish the response to subcutaneous histamine (BROCKLEHURST *et al* 1955) and to counter the effect of self-depression (SCHOU 1958 MILTHERS & SCHOU 1958). Mepyramine itself when mixed with $DF^{32}P$ and injected subcutaneously (11–12) depressed the absorption rate, in contrast with the findings of the above-named authors using sulphacetamide and morphine as the test substances. Even mice systemically treated with mepyramine absorbed $DF^{32}P$ at a slightly slower rate (10). Compound 48/80 administered locally at a concentration of 90 µg/ml (SCHOU 1958) decreased the incorporation of $DF^{32}P$ and this decrease was partially restored by prior treatment with mepyramine systemically (13 & 14). Similar results were obtained with histamine.

A group of 10 mice were given daily intraperitoneal injections of

Table 2

Influence of various substances on the rate of absorption of DF³²P in the mouse liver

Ser No.	Adjuvant to DF³²P		Dose of adjuvant mg/kg	No of animals	Activity in liver as % of control	s.e.m. ±
1	*DF³²P only*		–	15	100	3.9
2	Hyoscine	(50 µmol)	21.9	10	92	8.0
3	Hyoscyamine	(–)	17.8	10	58	7.4
4	*Homatropine*	(–)	17.8	10	62	8.7
5	Oxyphenonium (Antrenyl)	(–)	1.4	10	48	10.3
6	Atropine	(–)	17.4	15	61	4.5
7	–	(144 µmol)	50.0	15	46	5.7
8	– after mepyramine i. p.		17.4	10	66	6.8
9	– – –		50.0	5	46	18.2
10	*DF³²P only after mep. i. p.*		–	9	90	5.4
11	Mepyramine locally	(50 µmol)	20.1	10	59	8.1
12	– –	(125 –)	50.0	5	41	13.0
13	Comp. 48/80 locally		0.36	20	81	4.5
14	– – after mep. i. p.		0.36	20	91	3.7
15	Histamine locally		0.36	10	70	4.[illegible]
16	– after mep. i. p.		0.36	10	85	6.7
17	DF³²P only after hist. depletion		–	4	74	7.7
18	Atropine after hist. depletion		50.0	5	40	4.8
19	*DF³²P only to cortisone treated*		–	5	9	11.7
20	Atropine to cortisone treated		50.0	5	50	9.9
21	Morphine	(50 µmol)	18.8	10	66	5.2

Groups of 5 mice were injected with DF³²P either alone or mixed with the necessary compound. The volume injected into each mouse was uniformly 0.1 ml. Names of bases refer to the respective salts. All animals were killed 15 min after the injection of DF³²P. Mepyramine maleate, when used systemically was at 50 mg/kg intraperitoneally 45 min. before administering DF³²P. Depletion of body histamine was carried out by the 8-day schedule of FELDBERG & TALESNIK (1953). Cortisone acetate was given in 5 daily intraperitoneal doses at 10 mg/kg. Where more than 5 animals are indicated, the values of 2 or more series have been pooled.

compound 48/80 at the dose levels given by FELDBERG & TALESNIK (1953) for rats, viz. 1.5 2.0 2.5 3.0, 3.5 4.0, 4.5 5.0 mg/kg from the 1st to the 8th day to deplete them of their body histamine content. One mouse died on the first day. To the survivors distributed in 2 groups DF³²P and atropine-DF³²P mixture were administered on the 9th day. It was found (17 & 18) that there was a reduced uptake of DF³²P in both the atropinised and the control groups.

Table 3

Incorporation of $DF^{32}P$ in normal and histamine-depleted rats under various conditions.

N	Details	No of animals	Activity in liver as % of control
1	$DF^{32}P$ only	3	100
	With atropine at 17.4 mg/kg	3	6
3	As above after mepyramine i. p.	3	10
4	$DF^{32}P$ only to histamine-depleted	4	139
5	With atropine at 17.4 mg/kg to histamine depleted.	4	18
6	$DF^{32}P$ + mepyramine locally at 20 mg/kg	3	52
7	$DF^{32}P$ + 48/80 locally at 0.36 mg/kg	2	51
8	As above after mepyramine i. p.	2	49
9	$DF^{32}P$ + histamine locally at 0.36 mg/kg.	2	43
10	As above after mepyramine i. p.	2	52

Experiments 1 to 5 were carried out in one series on male rats, about 200 g. and experiments 6 to 10 were on another series with rats of about 250 g. Histamine depletion was carried out with Compound 48/80 by the 8-day scheme of Feldberg & Talesnik (1953). Two of the rats died on the first day. Systemical mepyramine was given at 50 mg/kg 45 min. before administering $DF^{32}P$. All injections were in a volume of 1 ml. $DF^{32}P$ with any adjuvant was injected subcutaneously under the loose skin on the neck, and the animals were killed 30 min. after the injections.

Cortisone administered intraperitoneally for 5 days before the injection of $DF^{32}P$-atropine mixture did not seem to have any enhancing effect on the rate of absorption (19 & 20)

Experiments on rats

Since the effect of atropine in retarding the rate of absorption has been found to be pronounced in the rat (Ramachandran & Ågren 1963b) some of the experiments carried out on mice with mepyramine and compound 48/80 were repeated on rats. Table 3 gives the results. It is seen that the depression effected by atropine was restored to a slight extent by mepyramine administered systemically before $DF^{32}P$-atropine (2 & 3 in table 3). There was a high uptake of $DF^{32}P$ much above those in the controls, in rats that had been depleted of their body histamine by compound 48/80 This is in contrast with the findings with mice (item 17 in table 2) Atropine-induced depression was only slightly restored but

not abolished in the histamine depleted rats (4 & 5 in table 3) As in the mice, mepyramine itself administered locally depressed the rate of absorption in rats (6 in table 3)

Experiments on guinea pigs

Guinea pigs are known to have a low skin histamine content of 3 μg/g, which is resistant to release, in contrast with rats and mice, which have a high skin histamine content of 40 μg/g, most of which is releasable (PERRY 1956) Guinea pigs also differ from rats in their susceptibility to allergies. It would thus be of interest to find if there is any retardation of absorption caused by atropine in this species. Some preliminary experiments carried out with a limited number of animals show that atropine does not have any appreciable effect in depressing the rate of absorption of DF³²P (table 4) Only with a high dose of atropine is some depression perceptible. The overall incorporation in all the atropinised animals in table 4 was about 90 / that of the controls.

Discussion

The results reported above indicate that atropine inhibits the rate of uptake of DF³²P in the mouse, but that the effect is less pronounced than

Table 4

Uptake of DF³²P in the Guinea pig liver in the presence of atropine.

N	Wt of animal g	Atropine mg/kg	Sacrifice tim min.	Activity in liver cpm × 10⁻³
1	360	-	15	57
2	350	17 4	15	66
3	410	–	30	151
4	410	17 4	30	162
5	420	–	60	236
6	410	17 4	60	207
7	420	–	30	383
8	420	30.0	30	302
9	270	–	30	794
10	270	50.0	30	516

Pairs of guinea pigs f about the same weight were chosen, and one animal was given DF³²P in 1 ml and the other the same volume of DF³²P containing the dded atropine. The umals were killed by beheading them under ether anaesthesia, and the livers were analysed in the usual way for radioactivity

in the rat. For instance in the presence of 50 µmol/kg of atropine the amount of DF³²P incorporated in the rat liver was as low as 6 / in 30 min. (table 3 item 2) whereas it was of the order of 60 / in the mouse under similar conditions (see table 1 at the 20 mg/kg level for 30 min or item 6 in table 2 for 15 min) A possible explanation is that the mouse is known to metabolise atropine at a much faster rate than the rat. GOSSELIN *et al* (1955) found that about 70–80 / of the injected atropine was excreted in the mouse in 6 hrs. in the rat about half of the quantity remained un excreted even at 48 hrs This species difference is also interesting in rela tion to the phenomenon of "self-depression" since the rat and the mouse have about the same skin histamine content of 40 µg/g (PERRY 1956), most of it in a releasable form.

The mixture of atropine with DF³²P results not only in a reduced rate of uptake of the latter but also in an overall reduction of the amount absorbed by the liver (fig. 1 48 hr values for liver) Since DF³²P and atropine do not mutually react (unpublished results), the reduction in uptake may be due to formation of a depot of DF³²P at the site of injec tion, allowing more scope for the detoxicating enzyme, DFPase, to act (RAMACHANDRAN & ÅGREN 1963a) It is seen from fig. 1 that the liver as a single organ absorbs more DF³²P than the combined total in the other three organs. A reduction in the total uptake effected by atropine may thus have a practical significance for the mode of administering drugs with atropine as an adjuvant. A case in point is the administration of oxime antidotes in anticholinesterase poisoning, when massive doses of 24 to 48 mg of atropine may be required for a person in one day (GROB 1963) The mode of administration of the oxime, either separately or with atro- pine at a high concentration, may make a difference to the absorption and elimination of the oxime, depending on whether atropine exerts its inhibitory effect in the human subject.

The mechanism of retardation in the absorption rate is thus worth study but the few experiments carried out by us to investigate how far atropine induced inhibition is a part of the phenomenon of self-depression have led only to inconclusive results. The fact that the atropine effect is pronounced in two species alone, the rat and the mouse, which have a high content of skin histamine, and that it is absent from the guinea pig, which has a low skin histamine, points strongly to histamine playing a role in this phenomenon On the other hand, the relatively insignificant influence of mepyramine and histamine depletion by compound 48/80 in countering the atropine effect indicates that atropine and similar com- pounds may have an effect *per se* apart from the relatively minor retarding effect mediated by the liberated histamine.

The effect of mepyramine itself in reducing the absorption rate of

DF³²P is a further point to note since this drug is found to enhance the rate of absorption of sulphacetamide (SCHOU 1958) and morphine (MILTHERS & SCHOU 1958) It has been verified that DF³²P and mepyramine do not mutually react (unpublished results)

Summary

The rate of absorption of subcutaneously injected DF³²P in mice was reduced by the presence of atropine in the test solution The extent of retardation depended on the concentration of atropine, within certain limits The degree of the reduction in uptake by the 4 organs tested, viz., the liver the kidney the lungs and the brain, was different, the incorporation of DF³²P in the lungs remaining relatively unaffected by the presence of atropine in the injection fluid.

The retarding influence of atropine was less pronounced in the mouse than in the rat. A few experiments on guinea pigs showed that atropine was without retarding effect in this species Besides atropine, many atropine-like substances had a retarding effect in mice, the most effective being oxyphenonium bromide (antrenyl ®) The atropine-induced retardation was not countered by the prior depletion of body histamine by compound 48/80 or systemic administration of mepyramine.

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Table 2

Actions of metropin and U247–42 on gastric secretion in Shay rats. Intraperitoneal injection. In the three-hour experiment five animals to each dose, in the five-hour experiment four animals.

Substance	Period	Volume ml	Gastric contents pH	Titration value in meq pH 3.5	pH 9.2	Mydriatic activity 30 min	1,5 hrs.	3 hrs.	5 hrs.
Control	3 hrs.	12.3	1.15	0.921	1.058				
Metropin 2 mg	3 hrs.	1.7	3.08	0.025	0.071				
U247–42 1 mg	3 hrs.	2.0	4.36	0.000	0.043				
Control	5 hrs.	15.9	1.25	1.147	1.302	0	0	0	0
Metropin 2 mg	5 hrs.	4.8	2.19	0.143	0.252	55 %	39 %	13 %	2 %
U247–42 1 mg	5 hrs.	3.3	2.02	0.111	0.193	0	0	0	0
Metropin 2 mg + U247–42 1 mg	5 hrs.	0.8	4.56	0.000	0.033	63 %	70 %	47 %	30 %
Metropin 1 mg + U247–42 0.5 mg	5 hrs.	3.1	3.14	0.015	0.139	48 %	38 %	20 %	8 %

tropin was increased somewhat when U247–42 was given at the same time.

There is, in other words, no doubt that the three quaternary compounds, when administered intraperitoneally to Shay rats, have both a protective action against ulcers and an inhibitory action on secretion. The effect on the stomach is additive to that of a simultaneously administered anticholinergic substance, and an increased absorption of this possibly results.

Actions after oral administration.

The Shay rat test is less suitable for studying the actions of drugs given by mouth, though it has also been used for this. Protective actions of antacid and antipeptic agents introduced into the ligated stomach have been observed by SHAY *et al* (1947), KOMAROV *et al* (1950), GROVER & MAASKE (1953), and KETRILÄINEN & PAASONEN (1957). Salicylic acid compounds given in the same manner likewise have a protective action (PAULS *et al* 1948, KATZ *et al* 1949). CAHEN & TVEDE (1951) compared the actions of methantheline bromide and atropine after subcutaneous or oral administration to Shay rats and found that to obtain identical effects after the two forms of administration the oral dose of atropine had to be 3 or 4 times greater than the subcutaneous, whereas the oral dose of methantheline bromide had to be 14 to 15 times greater.

An experiment was conducted to investigate how a quaternary com-

pound is absorbed from the gastro-intestinal tracts of Shay rats *Metropin* was given partly by *mouth* one hour before the ligation or introduced into the ligated stomach immediately after the operation and partly injected into the caecum *during* the operation

As seen from table 3 the greatest effect was obtained when metropin was given direct into the intestine. The action was *not* less when the drug was introduced into the ligated stomach than when it had time to pass into the intestine before the ligation. Some absorption thus seems to take place direct from the stomach.

Table 4 shows the action of *U247–42* administered partly into the caecum and partly into the ligated stomach. No effect could be demonstrated when 5 mg was introduced *into the* caecum. In the animals given the oral dose a marked effect was noticed on both ulceration and acidity However it remains possible that some of the drug administered had

Table 3

Action of metropin given to Shay rats by mouth or into the intestine.
Period of ligation nine hours. Four animals to each dose.

Administration	Dose in mg	Ulcer index	Gastric contents: Volume ml	pH	Titration values in meq.: pH 3.5	pH 9.2
Oral 1 hr before	10	2.3 × 100% = 230	9.5	1.36	0.50	0.69
Oral after ligat.	10	1.3 × 100% = 130	9.9	1.38	0.45	0.71
Oral after ligat.	5	1.7 × 100% = 170	9.7	1.32	0.44	0.62
Inject. into caecum	10	0.9 × 80% = 72	8.3	1.90	0.19	0.34

Table 4

Action of U247–42 introduced into the caecum or given by mouth to Shay rats
Period of ligation nine hours. Five animals to each dose.

Dose	Ulcer index	Gastric contents: Volume ml	pH	Titration values in meq.: Töpfer	Phenolpht.
Control	2.0 × 80% = 160	10.9	1.59	0.37	0.71
U247–42 5 mg in caecum	1.9 × 80% = 152	11.8	1.62	0.34	0.68
Control	3.4 × 100% = 340	10.5	2.51	0.70	1.59
U247–42 5 mg orally	1.0 × 80% = 80	9.2	2.62	0.21	1.13

been left in the stomach and that accordingly the effect may have been due to local action.

To be able to introduce the test substance as high up in the alimentary tract as possible because of the ligation the procedure employed was as described below After the ligature had been tied round the pyloric sphincter the test substance was injected just distally to the site of liga tion. Finally an extra ligature was applied distally to the site of injection.

As illustrated in table 5 5 mg metropin administered in this way had a marked effect, which was intensified by simultaneous injection of U247–42. The effect seemed to be due, at least in part, to increased absorption of metropin from the intestine, the mydriatic activity having been consider ably intensified.

Action on histamine treated Shay rats

ALPHIN & LIN (1962) compared Shay rats with rats having a chronic fistula and found the latter to be far more susceptible to histamine, presumably because the secretion was almost at a maximum in the totally ligated Shay rat stomach, so that any stimulating action was masked. The fact that no additional secretion takes place in the stomachs of Shay rats, when they are exposed to further stress during the period of ligation also proves that gastric secretion is at its maximum in these rats (MENGUY 1960 BRODIE *et al* 1962 SEGAL 1960) KYLE & WELLBAUM (1956), how ever found Shay rats to respond to large doses of histamine by increased secretion as well as ulceration.

Table 5

Actions on Shay rats of metropin and mixtures of metropin and U247–42 introduced into the duodenum. Period of ligation nine hours. Four animals to each dose.

Dose	Ulcer index	Gastric contents: V ml	Gastric contents: pH	Titration values in meq: pH 3.5	Titration values in meq: pH 8.2	Mydriatic activity: 20 min	Mydriatic activity: 2 hrs.	Mydriatic activity: 5 hrs.	Mydriatic activity: 9 hrs.
Metropin 5 mg	0.7 × 67% = 47	14._	1.45	0.623	0.772	13%	7%	0	0
Metropin 2,5 mg	2.6 × 100% = 260	17.3	1.68	0.551	0.770	0	4%	0	0
Metropin 5 mg, U247–42 4 mg	0	4.5	2.29	0.126	0.200	87%	97%	50%	35%
Metropin 2.5 mg, U247–42 2.0 mg	1.25 × 75% = 94	13.7	1.56	0.615	0.837	42%	15%	0	0

The action on histamine treated Shay rats is shown in table. 6 Subcutaneous histamine injected immediately after the ligation and again one and two hours later each time at a dose of 1 mg, increased the ulceration, so that after six hours grave ulcers were seen, often perforated. The values for acidity were therefore worth little. Doses of 5 mg metropin had a marked protective action. Further it was notable that the ulcers developed in the metropin-treated animals were localised in the glandular part of the stomach, as rarely occurred in untreated Shay rats. These may accordingly have been ulcers of different etiology against which metropin had no effect.

In doses of one-fourth and one-eight that of metropin U247-4? likewise had a protective action on these rats

In five Shay rats, which had had both vagus nerves transected at the same time as the ligation of the stomach, we found no ulcers nine hours after the ligation. This observation is in agreement with the findings of other workers as mentioned above in the review of the literature.

In exploratory experiments on vagotomised Shay rats even nine doses of 1 mg histamine each, injected subcutaneously during the ligation period, caused no gastric ulcers. In another experiment, therefore, seven doses of 2 mg histamine each were given during a nine-hour ligation period. It is shown in table 7 that this enormous dose of histamine could provoke ulcers in the vagotomised animals U247-42 doubtless had a certain protective action.

Table 6

Actions of metropin and U247-42 [illegible] histamine-treated Shay rats. Histamine chloride, 1 mg, administered subcutaneously immediately and [illegible] one and two hours after the operation. Period [illegible]f ligation six hours. Four animals [illegible] each dose.

Substance	Dose mg	Ulcer index		Gastric contents			
				Volume ml	pH	Titrating values in meq	
						Töpfer	Phenolpht.
Control		3.25	100 % = 325	about 17	1.26	about 1.1	about 1.4
Metropin	5.0	0.75	50 % = 37	8.2	2.10	0.154	0.300
U247-42	1.25	1.13	50 % = 56	8.4	1.60	0.417	0.590
U247-42	0.625	1.5	75 % = 113	12.5	1.28	0.85	1.032
							—

Table 7

Actio of U247–42 on vagotomised, histamine-treated Shay rats. Histamine, 2 mg, given subcutaneously seven times within the ligation period of nine hours. Each batch comprised five animals.

Dose	Ulcer index	Gastric contents			
		V lume ml	pH	Titration values in meq.	
				Töpfer	Phenolpht
Control	1.6 × 100 % = 160	7.2	2.59	0.09	0.207
U247–42 2.5 mg i.p.	0.1 × 20 % = 2	5.6	3.20	0.000	0 120

Action on Shay rats given gastric juice

A fair proportionality was found in the Shay rat experiments between the secretion-inhibitory action of a drug and its protective action against ulceration. The anticholinergic substances are supposed to act by blocking the parasympathetic nervous system, but this cannot be the mechanism of action of the quaternary non-anticholinergic substances. It was therefore thought to be of interest to compare the protective actions of the two types of drug on the stomachs of Shay rats given gastric juice into the ligated stomach.

The experiment was carried out as described below Gastric secretion was collected from Shay rats killed six hours after ligation. After centrifugation, there was from six rats a total amount 77 ml of pH 1.30 This was stored in refrigerator until the next day when the Shay test was performed on 12 rats. After ligation the animals received either 0 9 % sodium chloride, 5 mg metropin or 2 mg U247–42 intraperitoneally When fully conscious after the anaesthesia, they received orally by rubber tube, 2 ml of the gastric secretion and then 2 ml of water This was repeated about two and about four hours later and the animals were killed after seven hours of ligation. The results are given in table 8 The control animals were then in a poor condition Perforation was seen in most, and the gastric contents were so filled with blood that values for acidity were worthless. It is seen that both metropin and U247–42 had protected against the corrosive action of administered gastric secretion Metropin at a dose of 5 mg had a somewhat stronger effect than 2 mg U247–42. It is remarkable that the pH of the gastric contents from all the treated animals was higher than that of the secretion given, and that the volumes found in the stomachs were less than the 12 ml administered by mouth during the experiment.

Table 8.

Actions of metropin and U247–42 on Shay rats given gastric juice by mouth. Period of ligation seven hours. Four animals to each dose.

Dose	Ulcer index	Gastric contents: Volume ml	pH	Titrating values in meq. Töpfer	Phenolpht.
Control	4 0 × 100% = 400	–	–	–	
Metropi 5 mg i.p.	0.25 × 50 / = 12	8.5	1 41	0.158	0.235
U247–42 2 mg i.p.	0.75 × 75% = 56	10.0	1.89	0.232	0.337

Discussion

The quaternary non-anticholinergic compounds were found to have a secretion-inhibitory as well as a protective action against ulceration in Shay rats. They seemed also to be capable of stimulating the absorption of quaternary anticholinergic compounds from the gastro-intestinal canal. The drugs had a protective action on histamine treated Shay rats and on histamine-treated vagotomised Shay rats. Further protective action was noticed against the corrosive action of gastric secretion introduced into the ligated stomach. The anticholinergic drug metropin was found to have a similar action. Besides its action *via* the autonomous nervous system, this drug must therefore also be assumed to have other ways of protecting the animals

As stated in the introduction, most workers are of opinion that the ulcers in Shay rats develop by corrosion of the parts of the stomach not normally exposed to strong acids. Although several workers have employed the Shay test as a basis for ascertaining the actions of new drugs, they do not all agree in their interpretations of these experiments.

LAMBLING *et al* (1953) have advanced the hypothesis that the development of ulcers in Shay rats is a stage in a diffuse visceral disease resulting from the irritation of the pyloric sphincter As evidence in support of the view that the attack by pepsin and hydrochloric acid is not the essential factor it is stated, among other things, that atropine only protects against ulcers when given in much larger doses than those required to prevent secretion and that antihistamines protect without inhibiting secretion

Besides there being these two theories of the pathogenesis of ulceration in Shay rats, other workers have pointed out the importance of the electrolyte loss through the gastric secretion (KOWALEWSKI *et al* 1954)

and of the vasomotor reactions caused by the ligation (DEBRAY *et al* 1950 GATI *et al* 1961 LOZZIO *et al* 1961)

SEGAL *et al* (1952) and HAROUTUNIAN *et al* (1952) noticed that in general toxic actions on the animals, certain drugs can protect the stomach.

A drug that by a known mechanism reduces the secretion of gastric juice and prevents ulceration can be compared quantitatively with well-known drugs by the Shay test On the other hand, the test has been found unsuitable for solving the problem of what is the mechanism by which these quaternary non-anticholinergic drugs inhibit secretion.

Summary

The actions of three quaternary compounds, benzyl-tri-n-amyl-ammonium iodide, benzyl-(β-propoxyethyl)-ammonium iodide, and benzyl-(β-butoxyethyl)-ammonium bromide have been studied in Shay rats and compared with that of an anticholinergic drug (metropin ®)

The test substances had an inhibitory action on the secretion of gastric juice as well as on ulceration, either given intraperitoneally or introduced direct into the gastro-intestinal canal An inhibitory action was also obtained in vagotomised and in histamine-treated Shay rats.

The three quaternary compounds have no spasmolytic actions or any effect on the autonomous nervous system that can explain the inhibitory action on Shay rats.

The results have been discussed in relation to the different views previously advanced about the pathogenesis of the ulcers in Shay rats.

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Gastric Secretion of Rats after Test Meals

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The function of the stomach is studied clinically by giving a test meal and subsequently aspirating it. This procedure has been employed to a small extent in animal experiments

THORNTON & CLIFTON (1959) devised a method for use on rats It was based on clinical experiments by HUNT & SPURREL (1951), and it was later employed by VALBERG & WITTS (1961) in a somewhat modified form.

This is the only method in use that leaves the animals intact As they suffer no harm of any kind, the animals can be used more than once, which renders cross-over tests possible

Methods

Procedure

Female rats weighing 150–225 g were used. T secure empty stomach and sufficient hydration of the anim ls, these had been placed 24 hours earlier in wide-meshed wire cages with free access to a solutio containing 5 % glucose and 0.4 % sodium chloride.

A rubber catheter Nelaton no 9, was used for oral administration. Whereas THORNTON & CLIFTON (1959) worked with trained animals, which offered no resistance to the introduc tion of the catheter into the stomach, VALBERG & WITTS (1961) used immobilisation and llowed the catheter to be introduced despite resistance from the animals. By placing our rats on the wire cages and taking firm hold of their necks, we had no particular difficulty in introducing the catheter lubricated in dvance with glycerine, into the stomach. Only few animals had to be discarded after the first attempt, because they would not submit to the treatment. After short training, only a light hold of the back of th neck was required.

The stomach was washed first with 5 ml f boiled water t 37° which was aspirated quantitatively and then with 2½ ml, which was likewise aspirated. As the animals had drunk fairly large quantities of the glucose-saline solution during the 24-hour preliminary period, food residues ere rarely present to any appreciable extent in the stomach. Next 5 ml of the test meal, heated to 37° were introduced into the empty stomach. The test meal was a

solution of 15% crystalline sucrose, with phenol red added in an amount of 0.1 mg/ml and with the pH adjusted to about 6.5 by addition of 0.1 N H_2SO_4. Histamine was given immediately after this, and the animals were returned to the cages without access to water or food. Exactly 45 minutes later the gastric contents were aspirated, and the stomach was washed with 5 ml of boiled water heated to 37° which was aspirated quantitatively. The gastric contents plus washout were centrifuged, and their pH was measured with glass electrode. Titration was then performed with an automatic titrator (Electrometer) to the pH of the test meal by 0.01 N NaOH. The amount of acid was expressed as micro-equivalents of HCl. The amounts of phenol red in the test meal and in the aspirated fluid were determined spectrophotometrically at 560 mμ after dilution with a 0.5 M solution of $Na_2HPO_4 \cdot 2H_2O$ which fixed the pH at 9.86. The amounts of phenol red in milligrams could then be read from a standard curve based on a solution with 15% sucrose. The extinction conformed with Beer's law the plotted curve, with concentration in mg/ml as abscissa and E as ordinate, constituti g a straight line.

Calculations based on the results

As the stomach was not ligated, the amount secreted during the experimental period included that aspirated (contained in gastric contents (GC) plus final washout (FW)) as well as the amount passed through the pyloric sphincter (V p.p.).

V p.p is calculated from the equation

$$V\,p\,p = \frac{PRTM - (PRGC + PRWO)}{(CPRTM + CPRGC)\ 2}$$

where

PRTM = amount of phenol red in test meal,
PRGC = amount of phenol red in gastric contents,
PRWO = amount of phenol red in washings,
CPRTM = concentration f phenol red in test meal,
CPRGC = concentration f phenol red in gastric contents.

The approximation employed here was that the verage phenol red concentration in the fluid passing through the pylorus corresponded to the denominator of the fraction, namely half the sum of the initial nd the final concentrations in the stomach. Th concentration of phenol red in the gastric contents was calculated by dividing the total amount recovered by the volume found in the stomach.

The mount of HCl that had passed through the pyloric sphincter during the experimental period was calculated as the product of the volume of fluid passed and the average HCl concentration in the stomach. This having begun as zero, it became half of the final concentration.

Finally the total amount f acid was calculated as the sum of the amount aspirated from the stomach and that passed into the intestine.

The values set out i the tables illustrating the results are the pH in the stomach at the end of the experiment, the volume secreted during the experimental period, the amount of hydrochloric cid secreted, expressed as micro-equivalents, the volume of fluid passed into the intestine (V p p), a d finally the amount of phenol red recovered, expressed as percentage of that introduced into the stomach. The two latter values have been included to indicate whether the passage from the stomach to the intestine was normal or more or less obstructed.

The accuracy of the absolut values resulting from this mode of calculation may be open to question. The values seem, however to correspond fairly closely with those obtained on

rats by other procedures. At any rate, they can be used as basis for comparison of rats treated with different drugs.

Effect of histamine stimulation

The spontaneous acid secretion of the rat is sufficiently large to be measurable by the test meal method. As, however the main object of our experiments was to compare the actions of antagonists, the spontaneous secretion during 45 minutes was, in our view too low and irregular for this purpose.

It seemed most natural to use histamine as stimulant. Opinions differ considerably however about the sensitivity of rats to histamine. This is probably due to the many different methods used for measuring the secretion.

ROSE & BROWNE (1938) chose rats for investigating the distribution of histamine in the organism, because these animals are so resistant to the agent. ROE & DYER (1939) however demonstrated increased gastric secretion in anaesthetised rats given histamine or methacholine. FRIEDMAN (1943), on the other hand, noticed no action of histamine on gastric secretion. KOMAROV *et al* (1944) found histamine to have much less effect on rats than on dogs, cats or man, and LOEW (1947) stated that the rat is unsuitable for experiments with histamine because of the large doses required. HARKINS *et al.* (1947) could not provoke gastric ulcers in the rat by treatment with histamine in an oil-wax suspension. KYLE & WELLBORN (1956) had to use large doses of histamine to raise the secretion in Shay rats. NORDWIK & ANSON (1954) succeeded in raising the gastric secretion of rats in pentobarbital (mebumal) anaesthesia by injecting subcutaneously doses of histamine as large as 24 mg.

With a special technique, it has, however been possible within recent years to demonstrate the effect of much smaller doses of histamine on the rat stomach. GHOSH & SCHILD (1955) who perfused the stomachs of anaesthetised animals with a weak base, noticed the stimulating action of 50–100 μg histamine given intravenously. These were the same doses as sufficed for a method involving a dialysis bag inserted in the stomach (ANTONSSEN 1959). A stimulating action of physiological doses of histamine has also been demonstrated on the stomach in non-anaesthetised rats with gastric fistulae, as stated by IVY (1955). LANE *et al.* (1957) found 0.1 mg to have a stimulating action, and ALPHIN & LIN (1962) stated that maximum stimulation is obtained by 1–2 mg subcutaneously whereas larger doses are less active owing to the general toxicity of the drug. In comparative experiments on Shay rats, histamine administered in doses of up to 40 mg/kg had no stimulating effect (LIN *et al.* 1961). ADAMIEK & GROSSMANN (1963) likewise found a maximum effect of 2 mg histamine given subcutaneously to rats with gastric fistulae.

Using the test meal method, THORNTON & CLIFTON (1959) experimented with doses of histamine phosphate ranging from 0.5 to 8 mg subcutaneously. Here, too, a maximum effect was obtained with about 2 mg. VALBERG & WITTS (1961) tested doses ranging from 4 to 250 mg/kg subcutaneously and found increasing effects up to 25 mg/kg.

Results

Table 1 illustrates the actions of different histamine doses administered immediately after introduction of the test meal into the stomach.

Both subcutaneous and intraperitoneal doses are active. As, however subcutaneous injection seems to be painful and often causes necrosis, thus making the animals unfit for cross-over test, intraperitoneal administration was employed in the experiments reported below. As 3 mg had

Table 1

The stimulating actions of different doses of histamine in test meal experiments.

Dose of histamine	Number of animals	pH	Vol. ml	HCl μeq	Phenolred per cent	Vol. passed into intestine V p p ml
0.375 mg subc.	19	2.83	2.19	69.3	29	4.69
0.75 mg subc.	1	2.15	1.68	166.6	24	4.97
0.75 mg i.p.	23	2.21	1.94	120.0	25	4.72
1.50 mg i.p.	27	2.03	2.33	136.5	28	4.70
3.00 mg i.p.	29	2.21	1.80	111.1	30	4.30

no greater effect than 1.5 mg, the latter dose was chosen. The 136.5 μeq of hydrochloric acid secreted during the 45 minutes corresponds fairly closely to the amount to be read from the curve plotted by THORNTON & CLIFTON (1959). Change of histamine dose seemed to have no effect on the amount of fluid passing from the stomach to the intestine during the experimental period, the V p p as well as the recovered amount of phenol red being approximately the same in all the tests.

Table 2 shows mean values and standard errors calculated from 100 results on altogether 29 rats used as controls. These results were collected for an experimental period of about 6 months, the animals having only been used at intervals of 8–14 days and only about every third time as controls. The figures vary considerably, presumably owing in part to such factors as differences in temperature in the research room and differences in the intake of fluid during the preliminary period.

Table 2

Mean values and standard errors of 100 results achieved for 29 rats used as controls. Histamine dose 1.5 mg i.p.

	$\bar{x}$	s. e. m. $= \sqrt{\frac{\Sigma(x - \bar{x})^2}{n(n-1)}}$
V. I. secr. in ml	1.96	0.75
HCl secr. in μeq	118.1	13.7
pH	2.27	0.22
V. p. p. in ml	4.51	1.01

The experiments must therefore be carried out as cross-over tests. To compare the actions of two drugs we used, for example, 15 rats divided into three groups of five animals each. During three experiments, at intervals of 14 days, these groups received both drugs, as well as 0.9 / sodium chloride as control. Sometimes, however we stopped after two days. Further the value of the figures found was tested by calculating the t value (Student s test)

Actions of compound 48/80 and caffeine

The powerful histamine liberator compound 48/80, was injected subcutaneously at a dose of 0.5 mg at different times before the test meal. It is seen in table 3 that 48/80 caused a marked reduction in acid secretion by rats. Though the figures represent only a single experiment, they show plainly that acid secretion had almost ceased. The same dose of 48/80 has in other rats provoked gastric ulcers, which thus cannot have been due to increased acid secretion. The figures for phenol red and removal from the stomach show that the drug caused some retention. Retention of the secreted acid in the stomach may possibly be a concurrent cause of the harmful effect of the drug. If not, vascular factors must be assumed to be involved.

OWENS *et al* (1958) showed that histamine liberators reduce the volume of the stomachs of Shay rats as well as their acid secretion Simultaneous administration of atropine effected arrest of the acid secretion, but all the rats had gastric ulcers. The ulcer provoking effect was assumed to be due to action on the blood vessels of the stomach. MORENO & BRODIE (1962) noticed excessive ulcer formation in rats given the histamine liberator polymyxin. They showed that ulceration could be prevented by antihistamines and adrenaline, whereas the current anti-ulcer drugs had no effect. The action was not due to acid production, but to vascular

Table 3

Effect of 48/80 on the secretion in test meal experiments.
Single experiments on four animals to each dose.

Dose	pH	Vol. secr ml	HCl μeq.	Phenolred per cent	V p. p. ml
NaCl 1 ml s. c.	2 47	2.1	164.1	10	6 9
48/80 0.5 mg 15 m. bef re	6.1	0.4	0 9	39	3.7
30 m.	4.62	0.7	3.8	46	2.9
– 60 m. –	5 78	0.6	3 4	49	2.4
120 m.	5 72	1..	3 6	46	3.0

Table 4

Effect f caffeine on the secretion in test meal experiments.
Single experiments o four animals to each dose.
In test I the dose was given immediately bef re th test meal, in test II 30–50 minutes bef re and in test III 50–75 minutes before.

Test n	Dose	pH	Vol secr ml	HCl µeq	Phenolred per cent	V p. p. ml
	NaCl 1 ml i. p	2.61	1 7	87 9	19	5.1
I	Caffeine 1 mg i p	2.52	1.2	82.7	23	4.5
	– 2.5 – –	3 84	1.5	66.4	18	5.2
	– 5.0 – –	5 74	1.5	6.6	23	4 7
	NaCl 1 ml i. p	2.48	2.3	108.9	20	5.4
II	Caffeine 2.5 mg i. p	2.68	2.1	101.5	15	5.5
	– 5.0 – –	3 54	2.2	59 7	22	5.2
	– 10 0 – –	.71	2.4	56.7	45	3.5
	N Cl ml oral.	2.22	2 2	148.3	15	5.7
III	Caffeine 10 mg oral.	3.40	2.3	54.0	27	4.7
	– 20 – –	4.31	.1	19 6	42	4.7
	– 40 – –	4 05	1 2	18.6	12	5.3

changes. FRANCO BROWDER *et al* (1959) who likewise saw excessive ulcer formation in rats after administration of polymyxin, thought that this drug caused hyperaemia of the submucosa and congestion, with subsequent haemorrhage. The secretion was not considered as partaking in the pathogenesis. SELYE *et al* (1960) found stress ulcers in rats to differ from those provoked by 48/80 The latter could, in their opinion even be prevented by simultaneous exposure of the animals to stress. TSUKAMOTO (1961) laid bare the inside of the stomach and painted it with Congo red, a procedure that permitted direct observation of the acid secretion In this way he found histamine to be active at concentrations up to 10^{-5} whereas 48/80 had no effect. The failing stimulatory action of the latter on acid secretion is possibly specific to rats. SMITH (1953), for instance observed increased secretion after injection of 48/80 into chloralose anaesthetised cats

The results of experiments with *caffeine* as stimulant are shown in table 4 Contrary to expectation this drug had a markedly inhibitory effect on acid secretion after both intraperitoneal injection and oral administration. The re after 40 by mouth was, however possibly due to a general on, animals given this dose

Table 5

Antagonistic effects of subcutaneous and oral doses of metropin on histamine in test meal experiments.
d is the difference between the mean values, and t has been calculated by Student test.

Dose of Histamine	Dose of Metropin		pH	HCl µeq	Vol. secr ml	V p. p. ml	Phenolred per cent
		Control	2.83	69.3	2.19	4.69	28
	5 mg	Metropin	3.16	35.3	1.63	3.84	35
0.375 mg	subc.	d	0.33	34.0	0.56	0.85	7
		t	1.11	2.68	1.79	2.28	1.67
		p	0.1–0.5	0.01–0.05	0.05–0.1	0.01–0.05	0.1–0.5
		Control	2.05	136.5	2.33	4.70	28
	5 mg	Metropin	2.24	88.9	1.93	4.20	33
1.5 mg	subc.	d	0.19	47.6	0.40	0.50	5
		t	1.74	3.5	1.66	1.85	1.4
		p	0.1–0.5	0.01	0.10	0.05	0.1–0.5
		Control	2.20	136.0	1.87	4.80	25
	10 mg	Metropin	2.49	109.3	1.42	4.43	26
0.75 mg	oral	d	0.29	26.7	0.45	0.37	1
		t	2.34	1.14	1.35	0.98	0.35
		p	0.01–0.05	0.1–0.5	0.1–0.5	0.1–0.5	0.5
		Control	2.04	139.3	2.1	4.25	33
	10 mg	Metropin	1.88	115.2	2.80	4.25	33.7
1.5 mg	oral	d	0.16	24.1	0.68	0	0.7
		t	0.62	1.77	1.52	0	0.20
		p	0.5	0.1	0.1–0.5	0	0.5

dying on the next night. No effect was noticed on the amount of gastric juice secreted nor on that passing into the intestine.

The stimulating action of caffeine on the gastric secretion of man is unquestionable. The same is true for cats, in which, moreover, the agent potentiates the action of histamine (ROTH & IVY 1944a & 1945). On the other hand, these workers found no effect on dogs (ROTH & IVY 1944b).

Actions of anticholinergic compounds

For these investigations we used the quaternary compound (diphenylmetoxy)-ethyl-methyl-diethyl-ammonium iodide (metropin ®), whose effects have been described previously (ANTONSEN 1953, ANTONSEN & NIELSEN 1963), and atropine sulphate.

The antagonistic action of metropin against different histamine doses is shown in table 5.

Table 6

Effect of 5 mg atropine sulphate given subcutaneously

	pH	HCl µeq.	Vol. secr. ml	V p. p ml	Phenolred per cent
Control	2.20	136.0	1.87	4.80	25
Atrop. sulph 5 mg s. c.	2.67	64.7	1.27	4.46	25
d.	0.47	71.3	0.60	0.36	0
t	3.41	3.36	1.61	0.80	
P	0.01	0.01	0.1–0.5	0.1–0.5	

Subcutaneously 5 mg were given 30 minutes before the test meal, and 10 mg by mouth 90 minutes before.

The subcutaneous dose, which was sufficiently large to cause some mydriasis and some dryness of the throat, had a marked inhibitory action on the secretion of both acid and gastric juice. The effect was greater on the secretion of acid than on the amount of juice secreted, possibly because histamine acts fairly specifically on the former (KOMAROV et al 1944 SEGAL 1960) There was no essential difference between the antagonistic effects against the small and the large histamine dose. As might be expected of an anticholinergic compound, it inhibited the passage from the stomach. The V p p decreased, and the recovered amount of phenol red increased.

An oral dose of 10 mg metropin had a slighter action than the subcutaneous dose A greater antagonistic effect was noticed on the small dose of histamine than on the large one The passage from the stomach did not appear to be inhibited

The effect of 5 mg atropine sulphate injected subcutaneously is shown in table 6 This was a considerably larger anticholinergic dose than 5 mg metropin, and accordingly it had a more pronounced inhibitory action on acid secretion. It had no greater effect on the volume, and no inhibition of the passage from the stomach was seen.

The test meal method can be used for investigating the duration of the actions of antagonists on the stomach.

Table 7 illustrates the action of 5 mg metropin given intraperitoneally 1 3 5, 7 10, and 15 hours before the test meal. Each dose was immediately succeeded by administration of 1.5 mg histamine The test was carried through as a cross-over test for four experimental days on 32 rats

A marked inhibitory action was noticed on acidity and volume after 1 3 5 or 7 hours. The maximum effect was observed after 5 hours. This was surprising, because with intraperitoneal injection of metropin

Table 7

Duration of action of metropin in test meal experiments.
Metropin, 5 mg i. p., given at different times before histamine, 1.5 mg i. p. and test meal.

		Time in hours between administration and test-meal						
		Control	1	3	5	7	10	15
HCl μeq.	$\bar{y}$	119.2	104.7	55.4	43 7	58 0	107.5	102.4
	d	–	14.5	63.8	75.5	61.2	11 7	16.8
	t		1 09	5.87	7.08	5.30	0.87	1 10
	p		0.1–0.5	<0.01	<0.01	<0.01	0.1–0.5	0.1–0.5
pH	$\bar{y}$	2.15	2.36	2.59	[illegible].87	2.70	[illegible].04	1 80
	d	–	0 21	0.44	0.72	0.55	0.11	0.35
	t		2.51	4 76	6.05	3.87	1.00	1 67
	p		0 01–0 05	<0 01	<0.01	<0.01	0.1–0.5	0.1–0.5
Vol. secr ml.	$\bar{y}$	2.2	1 5	1 6	1 2	1.9	1.8	2.1
	d		0.7	0 6	1 0	0.3	0.4	0.1
	t		2 27	2.21	3.07	1 13	0.99	0.17
	p	–	0.01–0.05	0.01–0 05	<0.01	0.1–0.5	0.1–0.5	0 5
V p. p. ml.	$\bar{y}$	4 5	4 6	4.0	4.2	4.5	4.2	4.0
	d		0.1	0.5	0.3	0	0 3	0.5
	t	–	0.29	1 42	0.72	–	0 89	1.08
	p		0 5	0.1–0 5	0.1–0.5	–	0.1–0.5	0.1–0.5
Phenolred percent	$\bar{y}$	30	24	35	29	31	32	37
	d	–	6	5	1	1	2	7
	t		1.81	1 34	0.23	0 32	0.57	1.40
	p		0 05–0 1	0.1–0.5	0.5	0.5	0.5	0.1–0.5

a pronounced mydriatic effect was seen shortly after the injection and reached a maximum 15 to 30 minutes later In the test meal experiment the throat was found to be extremely dry at the time of introducing the rubber tube. This dryness lasted 1 to 3 hours after metropin had been given, but was not particularly noticeable later Thus the side-effects did not coincide with the maximum effect on the stomach. The Shay test affords no possibility of demonstrating this fact, as it only shows the final result of an influence lasting several hours.

Retention was at no time significant, but was most marked after 1 and 3 hours.

Significance of histamine antagonism.

Metropin thus doubtless has an inhibitory action on histamine stimulated acid secretion in rats. This agent having an appreciable anticholiner

Table 8

Comparison of actions of metropin and U247–65 in test meal experiments.
Metropin and U247–65 given three hours before 1.5 mg histamine and test meal.

		Control	Metrop 5 mg i. p	U 47–65 5 mg i. p.	Control	Metrop. 10 mg orally	U247–65 10 mg orally
HCl μeq	ȳ	89 6	48.3	38.2	107 0	95 0	67.8
	d	–	41.3	51 4	–	12.0	19.2
	t	–	2.31	2.97	–	0.92	1 41
	p	–	0 01–0.05	<0.01		0.1–0.5	0.1–0 5
pH	ȳ	2.59	2.85	3 20	2.29	2.33	2 38
	d	–	0.26	0 61		0.04	0 05
	t	–	1.23	1.88	–	0.45	0.96
	p	–	0.1–0.5	0.05–0 1	–	0.5	0.1–0.5
V i. secr ml	ȳ	1.8	1.5	1.5	1 5	1 6	1.8
	d	–	0 3	0 3	–	0.1	0 3
	t	–	1 23	1 38	–	0.47	1.03
	p	–	0.1–0 5	0 1–0.5	–	0.5	0.1–0.5
V p. p ml	ȳ	4 6	4 6	4.5	3 9	3 7	3.4
	d	–	0	0.1		0.2	0.5
	t	–	–	0.31		0.59	1 72
	p	–	–	0.5	–	0.5	0 1
Phenolred percent	ȳ	27	25	24	35	39	44
	d	–	2	3		4	9
	t	–	0.50	0 76	–	0.76	2.02
	p	–	0.5	0 1–0.5		0.1–0.5	0.05–0.1

gic and antihistaminic action it has been considered of interest to show which of these actions predominated in these tests.

To investigate the significance of the histamine antagonism a comparison was drawn with the analogous compound α methyl-metropin (code name U247–65) This compound shows histamine antagonism in isolated guinea pig intestine 1 7 times that of metropin, whereas its anticholinergic activity is no more than 0 8 that of metropin
Table 8 shows the effects of 5 mg given intraperitoneally and of 10 mg given by mouth three hours before the histamine test meal It is seen that U247–65 had a greater inhibiting action on acid secretion than metropin. The oral doses of both agents had considerably weaker actions, but the ratio was approximately the same as that for the intraperitoneal doses,

Table 9

Action of antazoline i test meal experiments.
Five animals to each dose.

Dose	HCl µeq	pH	Vol. secr ml	V p. p ml	Phenolred per cent
Control	135 6	1.90	2.1	4.2	33
Antazol. 2.5 mg subc.	167 1	2.07	2.6	4 7	28
5 0 – –	182.1	2 08	2.3	4 8	26

suggesting that the drugs are absorbed to a fairly equal extent from the gastro-intestinal canal

The increased inhibitory action that occurred simultaneously with a more pronounced histamine antagonism was rather surprising. According to the literature any inhibitory action of antihistamines on the gastric secretion has rarely been noticed in animal experiments.

The effect of antazoline (antistina ®), which has a two or three times greater antagonism to histamine action than metropin, but a weak anticholinergic action, is shown in table 9 The experiment was carried out as a single test only on 15 rats, but showed fairly clearly that the antihistamine had no inhibitory action. On the contrary acid secretion was found to be increased in the experimental animals.

Discussion

Unlike all other methods employed for studying gastric secretion in rats, the test meal method is performed on completely intact animals. This has the great advantage that anaesthesia and surgical intervention are avoided and that cross-over tests can be carried out.

The method gives information on the secretions of acid and gastric juice separately and also on possible retaining actions of test substances, because the extent of passage from the stomach to the intestine can be estimated.

Though the accuracy of the method of calculation employed is open to discussion, the values found seem to agree fairly closely with those obtained for rats by other procedures. As the experimental period extends over no more than 45 minutes, we must try to give the drugs at different intervals of times before the test meal in order to get an expression of the duration of the actions of these drugs.

To be able to study the actions of inhibitors, the secretion was stimu

lated by histamine This resulted in a mixed spontaneous and histamine stimulated secretion. The question whether histamine enters into the normal mechanism of secretion has been a subject for discussion (SMITH 1961 LIN *et al* 1962) and has probably not yet been finally clarified. Whether this is so or not, histamine may be regarded as the most physiological stimulant and antagonism to it as an adequate expression of the action of the drug concerned

The histamine liberator compound 48/80 having a marked inhibitory action on acid secretion the ulcer producing effect of this drug on rats cannot be due to increased acid secretion

The secretion stimulating action normally attributed to caffeine could not be demonstrated by the test method employed The doses given, both oral and injected, depressed gastric secretion. The only probable explanation of this is that the response of rats to the drug differs from that of man

As has been noticed with other test methods, the anticholinergic agents were found to have a marked inhibitory action. It is remarkable, however that such manifestations of anticholinergic action as mydriasis and dryness of the throat did not coincide with the effect on the stomach.

It is universally recognised that antihistamines fail to counteract the action of histamine on the stomach. In agreement with this, such a typical antihistamine as antazoline was found not to reduce secretion. Nevertheless, an increased effect of metropin was obtained by altering its structure so as to make its antagonism to histamine greater

It seems clear that the question of the effect of a new drug on the stomach can be answered to only a limited extent by experiments on isolated guinea-pig intestine.

Summary

The test method indicated by THORNTON & CLIFTON (1959) has been employed for testing the effects of various drugs on gastric secretion in rats.

The histamine liberator compound 48/80 and caffeine reduce histamine stimulated acid secretion

Anticholinergic agents have an inhibitory action, which reaches its maximum at a time that does not coincide with the other manifestations of their anticholinergic action, dryness of the throat and mydriasis.

An intensification of the histamine antagonism of an anticholinergic drug causes its inhibitory action to be increased The antihistamine antazoline, on the other hand, was found to stimulate gastric secretion.

The test method described has been found to be particularly useful. It can give information that has been found impossible to obtain on rats by other methods employed.

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Application of Test Meal Method for Investigating the Effect of some Quaternary Non-Anticholinergic Substances on the Rat Stomach

By

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(Received M y 21 1965)

In a previous paper (ANTONSEN 1965a), the effect of three quaternary non-anticholinergic substances on Shay rats has been discussed These substances caused a reduction of acid secretion as well as a protection against ulceration, though it was impossible to explain these effects from their other pharmacological effects.

To investigate how these substances affect the non-ligated stomach of normal rats, the test meal method described by THORNTON & CLIFTON (1959) was applied.

The three substances investigated were benzyl tri-n-amyl-ammonium iodide (code name U247–42), benzyl tri(β-propoxyethyl) ammonium iodide (code name U247–51) and benzyl tri-(β-butoxyethyl) ammonium bromide (code name U247–73)

As previously mentioned (ANTONSEN 1965a) these three substances have about the same protective effect on Shay rats, but they do not show any antagonist effect as well which might have explained the pharmacological effect mechanism of this protection.

For comparison, as in the previous experiments, the quaternary anticholinergic substance (diphenylmetoxy) ethyl-methyl-diethyl-ammonium iodide (metropin ®) was included.

Experimental Method

The modification of THORNTON & CLIFTON's (1959) method we have applied has been previously described (ANTONSEN 1965b).

Table 1

Effect of U247–42 and U247–51 administered subcutaneously and orally.
Histamine 1.5 mg administered intraperitoneally
T is average values for treated animals and C for control animals in same test, d is difference. t and p were calculated by Student's test.

Antagonist		C	T	d	t	p
	HCl µeq	136.5	50.8	83.7	5.8	<0.01
	pH	2.05	2.83	0.78	4.5	–
U247–42 1.25 mg s.c.	Vol. secr ml	2.33	1.87	0.46	2.0	0.04
	V p. p. ml	4.70	3.55	1.15	3.85	0.01
	Phenolred percent	28	42	14	4.1	–
	HCl µeq.	139.3	53.3	86.0	5.0	<0.01
	pH	*2.04*	*2.64*	0.60	*6.0*	–
U247–42 5 mg oral	Vol. secr ml	2.12	1.50	0.62	2.95	
	V p. p. ml	*4.25*	4.08	0.17	0.58	0.3
	Phenolred percent	33	7	1	0.19	
	HCl µeq	136.5	53.1	83.4	5.5	<0.01
	pH	2.05	2.88	0.83	3.5	–
U247–51 1.25 mg s.c.	Vol. secr ml	2.33	2.00	0.33	1.32	0.1–0.5
	V p. p ml	4.70	3.89	0.81	2.33	0.01–0.05
	Phenolred percent	28	38	10	2.39	
	HCl µeq.	136.8	52.6	84.2	5.69	<0.01
	pH	2.14	2.49	*0.35*	3.56	
U247–51 5 mg oral	Vol. secr ml	1.98	1.49	0.49	1.58	0.1–0.5
	V *p. p.* ml	*5.0*	3.7	*1.3*	4.71	0.01
	Phenolred percent	22	37	15	4.81	

All experiments were executed as cross-over tests, and the average results were compared by applying Student' s test.

Female Wistar rats were put into wide-meshed cages with free access to a solution containing 5 glucose and 0.4 % sodium chloride 24 hours before the experiment.

Results

Effect of U247–42 and U247–51 upon oral and subcutaneous administration

From table 1 we see that both U247–42 and U247–51 had a strong inhibitory effect on stomach secretion. Administered subcutaneously 1.25 mg had about the same effect as 5 mg administered orally. The subcutaneous injection caused considerable fluid retention in the stomach,

Table 2

Effect of 1.25 mg U247 51 administered intraperitoneally 1 3 5, 7 10 and 15 hours before histamine test meal. Histamine 1.5 mg i. p. Cross-over test on 32 rats. ȳ-average.

		Control	Hours between pre-treatment and test-meal 1	3	5	7	10	15
HCl µeq	ȳ	105.8	37.2	22.9	44 6	76.1	80.6	91.4
	d	–	68 6	82.9	61.2	29 7	25.2	14 4
	t	–	4.40	5 15	3.64	1 78	1.51	0.68
	p	–	<0.01	<0 01	<0.01	0.05–0.1	0.05–0.1	>0.5
pH	ȳ	2.33	3.46	4.50	3.30	3.04	2.50	0.15
	d	–	1 13	2.17	0.97	0.71	0.17	0.15
	t	–	3 74	5 06	5 64	2.62	1 48	0.99
	p	–	<0 01	<0.01	<0.01	0 01–0.05	0.1–0.5	0.1–0.5
Vol. secr ml	ȳ	2.4	3 6	2.4	2.3	2.3	2.0	1.9
	d	–	1.2	0	0.1	0.1	0.4	0.5
	t		4.27	–	0.51	0.43	1.84	2.65
	p	–	<0.01		>0.5	>0.5	0.05–0.1	0.01–0.05
V p. p. ml	ȳ	4.8	2.1	5 1	5.5	5.2	5.1	4.9
	d		2.7	0.3	0.7	0.4	0.3	0.1
	t		8 01	0.91	1.64	1 18	0.87	0.32
	p		<0.01	0.1–0.5	0.1–0.5	0.1–0.5	0.1–0.5	>0.5
Phenolred percent	ȳ	27	67	24	18	22	21	23
	d		40	3	9	5	6	4
	t	–	8.58	0.78	2.28	1.24	1.54	1.06
	p		<0.01	0.1–0.5	0.01–0 05	0.1–0.5	0.1–0.5	0.1–0.5

an effect also shown by U247–51 administered orally but not by U247–42.

These substances are, however not suitable for subcutaneous injection, as they cause irritation and pain at the site of injection. Excitation affects the animals, so that more force than normal must be used to insert the rubber catheter and the test will thus not take place under physiological conditions. A sympathomimetic effect of the substances may contribute to the excitation, as they cause an adrenaline like pressor effect when administered intravenously to anaesthetized animals.

Duration of the effect of U247–51

An investigation of the duration of the effect on intraperitoneal injection is illustrated by table 2. Doses of 1 25 mg were given 1 3 5 7 10 and

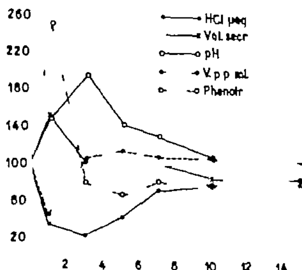


Fig. 1 Effect f 1.25 mg U247–51 dministered intraperitoneally 1 3 5 7 10 and 15 hours before histamin test meal.
Abscissa Time in hours. Ordinate the values for the treated animals as percentages of those for the co trol animals investigated simultaneously

15 hours before the histamine test meal. The test was executed as a cross-over on a total of 32 rats for four days of experiment. The maximum inhibitory effect on the acid secretion was seen after three hours, but there was also a significant effect after both one and five hours. We found a short lasting stimulation of non-acid secretion after 10 and 15 hours there was some lowering of this secretion There was also strong, though brief retention of the stomach contents, later on replaced by some increase in the rate at which the contents leave the stomach.

On fig. 1 the values for the treated animals were expressed as percentages of the values for the control animals, treated simultaneously

Comparison between the effects of U247–51 and U247–73 administered intraperitoneally

The effects of 1 mg U247–51 and U247–73 administered intraperitoneally one hour before the histamine test meal, are given in table 3

As shown in table 1 there was no essential difference between the effects of U247–42 and U247–51 However here U247–73 clearly differs. U247 73 had a stronger inhibitory effect on the acid secretion than U247–51 but lacked completely the violent retention effect of this substance Compared with the control animals, there is here even a slightly increased rate of passage into the intestine After U247–51 there is an increase of the volume after U247–73 though a faint reduction.

Table 3

Comparison between the effect of 1 mg U247 51 and 1 mg U247–73 administered intraperitoneally one hour before histamine test meal. ȳ-average Cross-over test on 15 rats.

		Control	U247 51 1 mg i. p.	U247 73 1 mg i. p
HCl μeq.	ȳ	92.1	35.7	6.7
	d	–	56.4	85.4
	t	–	3.36	6.37
	p	–	<0.01	<0.01
pH	ȳ	2.65	3.71	5.39
	d	–	1.06	2.74
	t		2.01	6.36
	p		0.05–0.1	<0.01
Vol. secr. ml	ȳ	2.3	3.1	1.9
	d	–	0.8	0.4
	t	–	2.06	1.12
	p		0.05–0.1	0.1–0.5
V p p. ml	ȳ	4.9	2.3	5.2
	d	–	2.6	0.2
	t		4.88	0.60
	p	–	<0.01	>0.5
Phenolred percent	ȳ	26	64	15
	d	–	38	11
	t		5.31	2.41
	p	–	<0.01	0.01–0.05

Comparison between the effects of U247–51 and metropin administered intraperitoneally

On figures 2 and 3 a comparison is shown between the effects of U247–51 1.25 mg and metropin 5.0 mg, administered intraperitoneally at various times before the histamine test meal. The maximum acid inhibitory effect occurred somewhat later after metropin than after U247–51 and at no time was there any increase of non-acid secretion after metropin, as we see from U247–51. The brief strong retention effect of U247–51 was also characteristic here. The figures for V p p. and recovered phenol red after metropin at no time differed from those for the control animals.

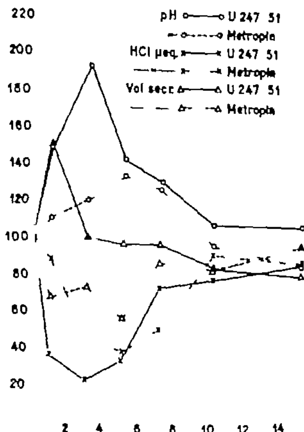


Fig. 2. Comparison between effect on acid secretion of 1.5 mg U247–51 and 5.0 mg metropin, administered intraperitoneally 1, 3, 5, 7, 10 and 15 hours before histamine test meal. Abscissa and ordinate as in fig. 1

Comparison between the effects of U247–51 and metropin administered orally

Although the quaternary non-anticholinergic substances in these tests as well had an inhibitory effect, definitely there are considerable differences between their effects and the effects of an anticholinergic substance.

For this reason, it might be of interest to investigate how a mixture of metropin and U247–51 administered orally at the same time, would affect the stomach.

The doses, dissolved in 2 ml water administered by means of a rubber catheter into the stomach of the fasting rats, were metropin 5 mg, U247–51 2.5 mg and a mixture of metropin 2.5 mg and U247–51 2.5 mg.

The results, expressed as percentages of the values for the control animals included at the same time, are shown in figures 4 and 5. The effect of metropin on acid secretion is slight and can only be demonstrated

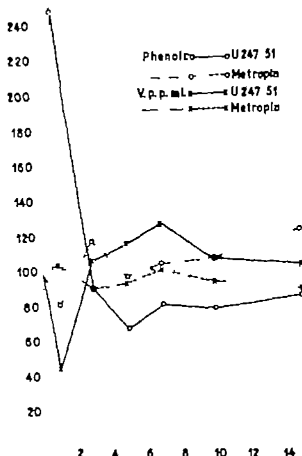


Fig. 3. Comparison between effect on gastric retention of 1.25 mg U247–51 and 5 0 mg metropin administered intraperitoneally 1 3 5, 7 10 and 15 hours before histamine test meal.
Abscissa and ordinate as in fig. 1

after one and three hours. The effect of U247–51 was considerably stronger and was significant after one, three and five hours. The effect of the mixture is only greater than that of U247–51 after one hour otherwise they are about the same. We did not notice any effect on the volume either of the mixture or of the substances separately The retention effect of U247–51 which only is seen after one hour was not modified by the admixture of metropin Everything considered, the two substances seem to have operated more or less independently from each other as U247–51 had the stronger effect, the curves for the mixture were most like the curves for this substance.

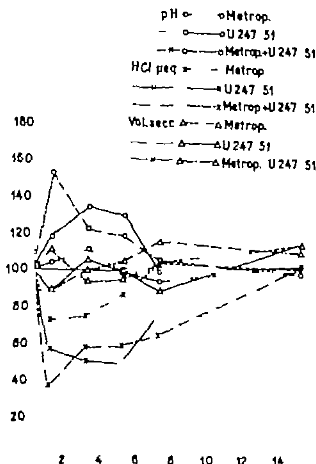


Fig. 4 Comparison between effect on acid secretion of 2.5 mg U247 51 and 5.0 mg metropin, plus mixture of 2.5 mg U247–51 and 2.5 mg metropin, administered orally 1 3, 5 7 and 15 hours before histamine test meal.
Abscissa and ordinate as in fig 1

Discussion

Experiments by the test meal method have shown the inhibitory effect that these quaternary non-anticholinergic substances have on the ligated rat stomach also to be demonstrable on intact rats. However we see considerable differences from the effect obtained with an anticholinergic substance in spite of the strong inhibitory effect on acid-secretion, the quantity of liquid secreted is not reduced, and we may even see an increase

The two substances U247–42 and U247–51 caused a strong retention of liquid in the stomach, whereas this was not observed with U247–73

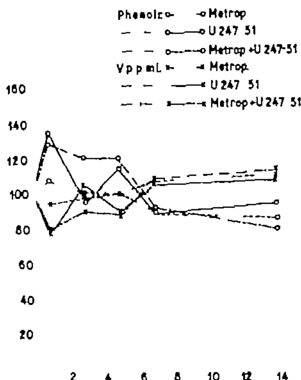


Fig 5 Comparison between effect on gastric retention of 2.5 mg U247 51 and 5 0 mg metropin plus mixture of 2.5 mg U247–51 and 2.5 mg metropin, administered orally 1 3, 5 7 and 15 hours before histamine test meal.
Abscissa and ordinate as in fig. 1

Although the substances have a typically quaternary structure, a relatively strong effect is obtained after oral administration they are not suitable for injection, because of their locally irritating effect. Whereas some of the Shay rat experiments (ANTONSEN 1965a) *might seem to indicate* that these substances can increase the absorption of a quaternary anticholinergic substance from the gastrointestinal tract, no alteration in the effect of the separate substances when administered simultaneously could be established in the test meal experiments.

As mentioned before, the substances are inactive in all *in vitro* experiments, with no ganglionic blocking or inhibitory effect on the autonomic nervous system. Administered intravenously to anaesthetized animals, a brief adrenaline-like pressor effect was caused by U247–42 and U247–51 but only to a less degree by U?47–73 possibly some kind of sympathomimetic effect may be involved in the effects they exert on the stomach.

Somewhat contradictory information is found in the literature about the action of sympathomimetic substances on stomach secretion (BAXTER 1934 LINDE 1950 CODE 1951 FORREST & CODE 1954 HARRIS 1956 PRADHAN & WINGATE 1962) However it is agreed that they have an inhibitory effect on acid secretion An increase in mucous stomach secretion has also been detected after these substances.

Summary

The effects on the rat stomach of three quaternary compounds, benzyl tri n amyl-ammonium iodide, benzyl-tri-(β-propoxy-ethyl) ammonium iodide and benzyl-(β-butoxy-ethyl) ammonium bromide compared with an anticholinergic substance (metropin ®), have been investigated by the test meal method of THORNTON & CLIFTON (1959).

All three substances had a clear inhibitory effect on acid secretion on both parenteral and oral administration Two of the substances brought about an increase of the non-acid secretion and caused the passage of the stomach contents into the intestine to be delayed

The duration of the effect of the substances has been investigated by administration at various times before the histamine test meal. The pharmacology of their mechanism of action is not known, but *possibly* a sympathomimetic effect is involved.

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Relative Effects of L-DOPA and Its Methyl Ester Given Orally or Intraperitoneally to Reserpine-Treated Mice

By

John D. Utley and Arvid Carlsson

(Received J ne 9 1965)

In laboratory animals parenteral administration of L DOPA results in central stimulation as well as peripheral symptoms These effects are probably due to decarboxylation of L DOPA to dopamine (DA)

Clinical studies have shown that DL DOPA given orally causes an increase in total plasma catecholamines, an increase in blood pressure, sinus bradycardia and certain psychic symptoms in patients pre treated with a MAO inhibitor but not in patients pre-treated with imipramine or a placebo (SCHILDKRAUT *et al* 1963 KLERMAN *et al* 1963) Oral DL DOPA did not alleviate mental depression (KLERMAN *et al* 1963), nor did it reverse drug-induced extra pyramidal reactions (MCGEER *et al* 1963), although the last-named workers found evidence that some DL DOPA was absorbed orally and increased blood pressure was seen in the one subject to whom an MAO inhibitor was given first. Other workers have found, however that oral and parenteral administration of DOPA alleviated akinesia or rigidity of parkinsonism or both (BARBEAU *et al* 1962 BIRKMAYER & HORNYKIEWICZ 1962).

Studies on intraperitoneal injection of the highly soluble L-DOPA methyl ester hydrochloride show that this compound reverses reserpine effects on cat-brain catecholamines and conditioned avoidance response, probably after being de methylated to L DOPA in the body (HANSON & UTLEY 1965) It was therefore of interest to compare the effects of L DOPA and its methyl ester hydrochloride when given orally and intra peritoneally to mice by measuring brain DA levels and by observing their behaviour

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Methods

Brain DA was determined by the fluorimetric technique of CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962). Animals were given 5 mg/kg reserpine intraperitoneally 20 hours bef re 400 mg/kg L DOPA or its methyl ester hydrochloride, calculated as L DOPA. No difference in bsorption was found whether oral L DOPA was given in aqueous suspension or dissolved in HCl. The methyl ester was dissolved in water. Intraperitoneal inject ons were made in saline.

Experiments were performed t eliminate possible fluorescence f the L-DOPA methyl ester The compound was found to be oxidized in both the ferricyanide procedure for noradrenaline and the iodine method for DA. With model extracts containing the methyl ester th compound was adsorbed o the resin and eluted with the DA fraction. Therefore part of each DA eluat was oxidized by means of ferricyanide with a methyl ester standard, an activating wavelength of 355 mμ and a fluorescent wavelength of 470 mμ. The resultant sample fluo escence value was converted to its equivalent fluorescence in the iodine method by oxidizing a methyl ester standard along with the DA estimations. The converted methyl ester fluorescence was subtracted from the DA fl orescence and found to contribute less than 1%.

Hearts were taken from some animals after 20 nd 30 minutes f methyl ester estimations and were found to contain o ly trace amounts of methyl ester

The 5-HTP decarboxylase activity was measured i ivo fter intraperitoneal injection of the L DOPA methyl ester 200 and 400 mg/kg, into reserpine-treated mice nd there is no significant difference from control values.

Results

Fig. 1 compares brain DA in mice at various intervals after intraperitoneal L DOPA and its methyl ester With L DOPA there is an initially higher peak after which the curves are similar

Fig. 2 shows that oral administration of the methyl ester causes a higher brain DA peak than an equivalent dose of oral L DOPA, but afterwards the curve is similar

Fig. 3 compares brain DA after different doses of L DOPA and its methyl ester given intraperitoneally or orally Both compounds result in high brain DA levels 30 minutes after intraperitoneal injection, but the methyl ester is apparently better absorbed by the oral route than L DOPA.

Peripheral and central effects were observed after both L DOPA and its methyl ester whether given orally or intraperitoneally in doses of 200 mg/kg or higher These effects began 10–15 minutes after the compounds were given and ended about 45 minutes afterwards.

Discussion

Since most substances are absorbed easily from the peritoneal cavity the methyl ester of L DOPA probably enters the blood stream intact and is then hydrolysed, possibly in the liver From the shapes of the two brain

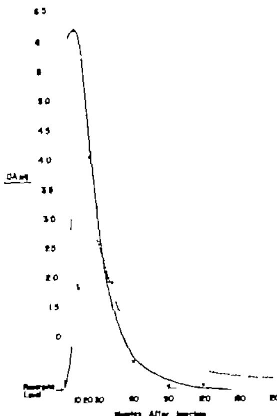


Fig. 1 Brain DA levels reserpine-treated mice after 400 mg/kg intraperitoneal DOPA (O———O) L DOPA methyl ester (●— — —●) calculated as L DOPA

DA curves in fig. 1 it may be concluded that L DOPA itself is even more easily absorbed from the peritoneal cavity than its methyl ester or that all the ester is not split immediately The results in fig. 2 show that absorption of orally administered L DOPA is poor relative to that given intra peritoneally The orally administered methyl ester however is absorbed better than L DOPA indicating that the methyl ester is absorbed intact. Fig. 3 shows that the difference in intestinal absorption is more pronounced after high than after low doses of the drugs.

Summary

Both L DOPA and its highly soluble methyl ester hydrochloride were administered orally and intraperitoneally to mice pre treated with reserpine Brain dopamine rises faster after intraperitoneal L DOPA than after its methyl ester but faster after oral methyl ester than after L DOPA.

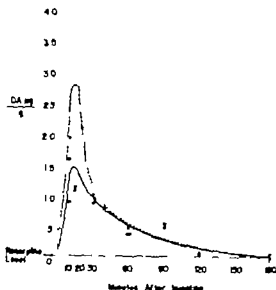


Fig. 2. Brain DA levels in reserpine-treated mice after 400 mg/kg oral L DOPA (O———O) or L DOPA methyl ester (● - - - - - - ●) calculated as L DOPA.

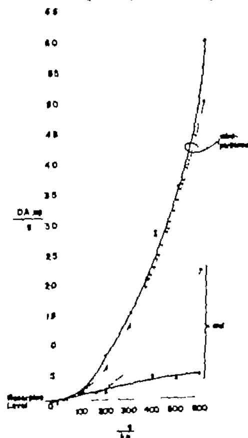


Fig. 3 Brain DA levels in reserpine-treated mice 30 minutes after various doses of L DOPA and its methyl ester given orally and intraperitoneally. The methyl ester was calculated as L-DOPA

Acknowledgements.

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Hyaluronidase as an Accelerator of Muscular Absorption of Water and Water-Soluble Compounds

By

Reidar B. Sund) and Jens Schou

(Received April 5, 1965)

In a previous paper (SUND & SCHOU 1964) the absorption from rat thigh muscle of the carbohydrates mannitol sucrose inulin (m w 3.000-4 000) and dextran (m.w 60 000–90 000) was reported. Their clearance rates (relative clearance) were inversely related to molecular size in a way that indicated the absorption process to be due mainly to diffusion.

In further experiments the absorption of these substances has been studied with hyaluronidase added to the injection solutions. Moreover the clearance rate of tritiated water in absence or presence of hyaluronidase was determined. The results, which are presented and discussed below show that the enzyme promotes the absorption of the various substances to an extent increasing with their molecular size. This finding is in qualitative agreement with results obtained in *in vitro* studies by OGSTON & SHERMAN (1961) and LAURENT *et al* (1963)

Materials and Methods

Test substances

Tritiated water ^{3}H-water of specific activity 250 µC/g, obtained from New England Nuclear Corp., Boston, USA

Radio-carbohydrates The same labelled mannitol-^{14}C sucrose-^{14}C methoxy-inulin-^{3}H and carboxyl-dextran-^{14}C as used previously (SUND & SCHOU 1964).

Hyaluronidase A batch of penetrase ® commercial purified and lyophilized testicular hyaluronidase obtained from Leo Pharmaceuticals, Copenhagen.

The injection solutions were prepared with glass-distilled water With hyaluronidase present, they contained 0.85 w/v sodium chloride, otherwise 0.9 %. Those of radio-water contained 62.5 or 125 µC/ml, those of radio-carbohydrates had the same concentration as previously (SUND & SCHOU 1964). Solid hyaluronidase was dissolved freshly on each experimental day and was added in a final concentration of 300 I.U./ml. This was a

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concentration above maximal, since even ½ f this concentration appeared to give the same results as those reported below A solutio used to determine the *exits of inulin and dextran from the blood* contained 1.,5 μC/ml dextran-carboxyl-^{14}C (21 mg/ml), 28μC/ml inulin-methoxy-^{3}H (1.5 mg/ml) and 0.9 % sodium chloride.

Animal experiments

The *absorption experiments* and the *analysis of the muscle samples* were carried out as described earlier (SUND & SCHOU 1964). Throughout the work, a constant volume of 16 μl was injected.

T exclude the possibility that the pron unced influence of hyaluronidase upon the dextran clearance could be in part due to splitting up of this polysaccharide by the enzyme, special experiment was performed A standard injection solution of dextran (21 mg/ml) was ncubated *in vitro* with hyaluronidase (300 I.U./ml) at 37° for 18 hours. Next, the enzyme was inactivated at 120° for 20 minutes. The slightly turbid solution was injected into rats in the usual manner Part of the same dextran solution, but without hyaluronidase, was carried through the same procedure, serving as control to the effect of the heat treatment upon the compound.

The *learance of inulin and dextran from the blood* was studied in male rats weighing 160 to 230 g. They were anaesthetized with sodium mebumal (pentobarbital) intraperitoneally (80 mg/kg), as in the clearance experiments, before double nephrectomy by the dorsal pproach. Then 100 μl of the solution mentioned was injected into the exposed femoral vein by means of an Agla ® micrometer syringe. Special attention was given to preventing leakage during and after the injection. Blood samples (250 μl each) were drawn from a tail vein into a heparinized plastic straw 5 15 30, 60, 120, 180 and 40 minutes after the injection. From single animals maximum number of 4 samples were drawn. To remove protein 100 μl plasma underwent zinc hydroxide precipitation (SOMOGYI 1930). After spinning, 150 μl clear supernatant from a sample were added to 4 ml of scintillation medium (BRAY 1960). The radioactivity f each sample was measured in a liquid scintillation counter (Isotope Developments LTD) For the estimation of dextran-carboxyl-^{14}C and inulin-methoxy-^{3}H in the sample, the screening method of OKIT et al. (1957) was used.

Results

Clearance of tritiated water

Fig. 1 shows a semilogarithmic plot of the percentage residual amounts of radio-water against time, when the compound was injected with or without hyaluronidase It is concluded that the clearance rate of radio-water is the same whether the "spreading factor" is present or not. The curved line has been drawn in the figure to give the best fit for all values. The water clears the muscle fairly rapidly at a rate similar to but slightly lower than, that of mannitol in the absence of hyaluronidase (cf SUND & SCHOU 1964)

Absorption of carbohydrates

The results from this experiment are tabulated in table 1 and are further illustrated in fig. 2 For comparison, values for mannitol and dextran in

Fig. 1 Clearance rate of tritiated water
16 µl of an isotonic solution containing 62.5–125 µC tritium oxide/ml and no (solid circles) + 300 I.U. hyaluronidase/ml (open circles) was injected
Abscissa Time (minutes) after injection.
Ordinate Percentage amount unabsorbed. Logarithmic scale
Vertical bars indicate S.E.M. In the horizontal brackets are given the number of determinations.

the absence of hyaluronidase (from SUND & SCHOU 1964) have also been included in the figure. The fastest moving substances have been followed until about 10 % remains unabsorbed. Below this value the accuracy of the estimates is considered too low for quantitative comparison. The clearance rates of the various substances are inversely related to molecular *size, as previously noted (SUND & SCHOU 1964).*

Since even a semilogarithmic plot of the residual amounts against time shows curvature, the absorption rates cannot be compared by a single "clearance constant" (KETY 1949). It seems for our purpose justifiable, however, to calculate a "clearance factor" for the various experimental periods. This factor (F) is defined as

$$F = \frac{(R_1 - R_2)\ 100}{R_1} \cdot \frac{1}{t - t_1}$$

where R_1 = residual amount at time t_1 and
R_2 = – – – – t_2 ($t_2 > t_1$)

Table 1

Absorption of various sized carbohydrates in presence of hyaluronidase.
16 μl samples of the solutions listed below all containing 300 I.U penetrase ®/ml and 8.5 mg sodium chloride/ml, were injected. Results are given as the mean percentage amount ±S.E.M. remaining at specified periods after the injection. With the exceptions stated, all mean values come from 4 experiments. The experimental result were compared by t-test with the control figures given by SUND & SCHOU (1964) in table 2.

	2½ or 3 min.	5 min.	7½ min.	15 min.	30 min.	60 min.
Mannitol-^{14}C	2½ min.	1)3)				
2.1 mg/ml	42.5±5.0	24.5±2.4	–	8.1±2.5	–	–
Sucrose-^{14}C						
0.19 mg/ml	–	27.6±3.5	–	8.7±1.3	–	–
Inulin-methoxy-^{3}H	3 min.				2)4)	
1.9 mg/ml	82.1±2.2	–	55.8±1.7	43.7±2.2 3)	16.5±1.2	–
Dextran-carboxyl-^{14}C						
21 mg/ml.	–	83.8±2.6 3)	–	67.5±1.8 4)	53.5±2.9 4)	35.6±4.6 4)

1) 5 expts. 2) 3 expts. 3) $P < 0.05$. 4) $P < 0.001$

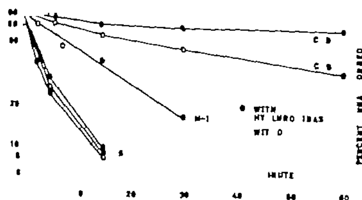


Fig. 2. Absorption rates in the presence (open circles) and absence (solid circles) of hyaluronidase.
M = mannitol, S = sucrose, M-I = methoxy-inulin, C-D = carboxyl-dextran.
Abscissa Time (minutes) after injection.
Ordinate Percentage amount unabsorbed. Logarithmic scale.
The curves are drawn on the basis of the figures contained in table 1 of the present paper and in table 2, SUND & SCHOU (1964).

Mathematically the factor expresses the average absorption rate (%/min.) in the period ($t_1 - t_2$) when the amount existing at the beginning of the period is taken as 100%. We are well aware of the inexactness of this factor, but it represents a usable mean for a semiquantitative comparison of absorption rates. The ratio (F_{Hy}/F_O) of the factor obtained for each compound in the presence (F_{Hy}) and absence (F_O) of hyaluronidase is shown for the various experimental periods in table 2. The essential finding is that there appears to be a progressive increase in the absorption-enhancing effect of the enzyme with increasing molecular weight of the absorbed substance. Thus, the absorption rate of mannitol is increased only by about 10% during the first 5 minutes after injection, but that of sucrose and inulin is increased by about 20 and 30% respectively and that of dextran about 100%. Contrary to the findings for the other compounds, the enhancing effect on dextran absorption appears to increase with time.

Effect of hyaluronidase upon dextran in vitro

From 5 rats injected with dextran that had been incubated with hyaluronidase, the mean residual amount (±S.E.M.) 15 minutes after injection was 82.4 ± 1.7. Four rats injected with dextran that had been carried through the same procedure (without hyaluronidase) as a control gave a mean of 83.2% ± 2.2. The two results do not differ significantly from each other, nor did they differ from the mean obtained from 4 other rats (77.6% ± 1.7) injected with dextran that had been subjected to neither hyaluronidase treatment nor heat. It could therefore be concluded that splitting the dextran molecule by the enzyme was not the reason for the large enhancing effect on the absorption of dextran or for the increase of this enhancement with time.

Table 2

Ratio of clearance factors.

Clearance factors (cf. text) at the specified absorption periods have been calculated for the various carbohydrates, in the presence (F_{Hy}) or absence (F_O) of hyaluronidase. The ratio F_{Hy}/F_O indicates the increase in absorption rate caused by the enzyme. Calculations are based on values contained in table 1 of this paper and in table 2, SUND & SCHOU (1964).

	Period after injection			
	0–5 min.	5–15 min.	15–30 min.	30–60 min.
Mannitol	1 1	1.0		
Sucrose	1 2	1 1		–
Methoxy-inulin	1 3	1 4	1.2	
Carboxy-dextran	2.1	1 6	4.–	8.5

Clearance of dextran and inulin from blood to interstitial fluid

In this experiment the decline in radioactivity of the blood was followed after simultaneous intravenous injection of carboxyl-^{14}C-dextran and methoxy-^{3}H inulin into nephrectomized rats. The pooled results from seven animals are illustrated in fig 3 where the "volumes of distribution" for the two substances are plotted against time The "volume of distribution" as a percentage of the bodyweight (R) is a theoretical value calculated for each of the compounds on the basis of the amount (cps) of radioactivity (D) injected, the radioactivity measured at the time t for the given tracer in cps per ml of blood plasma (c), and the body weight (b w)

$$R_t = \frac{D \times 100}{c_t \times b\,w}$$

It expresses the percentage of the body weight that should serve as a solution medium for the substance with a concentration the same as that in the blood plasma, to account for the injected material, at a given time.

The results demonstrate that the applied preparation of dextran is partly cleared from the circulating blood. The clearance curve for dextran is mainly the same shape as the curve for inulin an indicator accepted as being distributed in the extracellular compartment, although dextran travels at a significantly slower rate Four hours after the injection the calculated volume of dextran distribution is about 15 / b w at a time when the concentration of dextran in the blood still decreases with a declining rate.

Discussion

Clearance of tritiated water

The net absorption of water from the injection deposit cannot be ascertained from the experiment described Probably this absorption proceeds at a slow rate because only small gradients in water activity and in osmotic and hydrostatic pressures, exist across the "absorbing membrane" The clearance of tritiated water is thus assumed to be for the most part due to the usual (physiological) *exchange of water* molecules, by diffusion and paracapillary circulation wich normally occurs between the interstitial and the circulating water

The clearance can be regarded as an *absorption of tritiated water* a compound foreign to the body Two observations were made in this connection. First, the absorption rate is not enhanced by hyaluronidase, i. e. neither filtration rate nor diffusion rate, appears to be increased This failure of hyaluronidase to affect the diffusion of the small water molecule is in complete agreement with the results obtained for the carbohydrates of various molecular sizes. Moreover this absence of effect on the flow of water suggests that hyaluronidase does not increase capillary

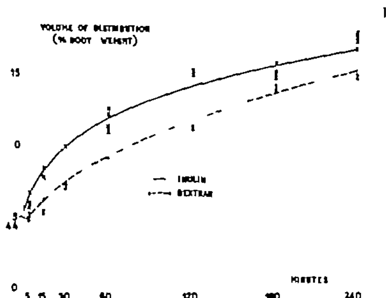


Fig. 3. Volume of distribution of dextran-carboxyl-^{14}C and inulin-methoxy-^{3}H in anesthetized rats. Pooled values from double tracer experiments on seven animals. The curves begin at the value 4.4 % body weight, which was experimentally determined as the plasma volume in mice (HVIDBERG, LANGGÅRD, SCHOU & SZPORNY 1964).
Abscissa: Time (minutes) after i. v. injection.
Ordinate: Dextran (● ● — — ● ●) and inulin (× × — × ×) compartments, expressed as percentage of body weight.

permeability. Different opinions have been held about this. However if such a mechanism was part of the pharmacological effect of hyaluronidase, the water flow probably would increase, (cf. USSING 1961). Secondly, in spite of a much larger diffusion coefficient in water (at 35° 3×10^{-5} cm^2 sec^{-1} according to WANG *et al.* (1953), compared to 0.8×10^{-5} for mannitol according to *International Critical Tables* 1929), the absorption rate of tritium oxide was similar to, but slightly lower than, that of mannitol (SUND & SCHOU 1964). This finding indicates, as might be expected, that a large proportion of the radio-water enters the cells within the injection zone, whereas mannitol does not.

Absorption of carbohydrates

Our results confirm and extend the observation of SCHRIFTMAN & KONDRITZER (1957) that hyaluronidase enhances absorption from muscle. The lack of effect of the enzyme on water clearance suggests that the increased rate of absorption is essentially due to an increased diffusion rate. It is reasonable to believe that the mucopolysaccharide barrier to diffusion, which is broken down by the enzyme, is formed by the inter

stitial matrix in the mostly loose intramuscular connective tissue. As would be expected from the high nutritional requirements of muscle fibres, this matrix has but a small restraining action upon the movement of small molecules such as those of mannitol. It is evident from table 2, however that the retarding effect increases with increasing molecular volume of the travelling substance. This observation is in qualitative accordance with results obtained by *in vitro* experiments with hyaluronic acid jellies (OGSTON & SHERMAN 1961 and LAURENT *et al* 1963)

From previous work (SUND & SCHOU 1964), when the same substances were studied without hyaluronidase, it was concluded that diffusion was a main driving force for their absorption. This conclusion was derived, firstly from the observation that the clearance rates were inversely related to molecular size. Secondly the ratio of the "clearance factor" (within a specified period) to a reasonable free diffusion coefficient ($D \times 10^6$ 37°) was fairly constant for the various substances. There was, however a tendency to an increase in the ratio with increasing molecular weight. Thus, for the first 5 minutes the ratios named from mannitol to dextran were 1 6, 1 7 2.1 and 3 0 In these further experiments figures of 1 7 1.9 2.8 and 6.8, respectively were found.

The deviation from a single value within each set of results may be explained if the observed absorption rates are the sum of two rates, the first one due to diffusion and influenced by hyaluronidase to an extent determined by the size of the diffusing compound. The other comes from solvent drag, does not depend upon molecular size and is not affected by hyaluronidase This is in effect the same view as that put forward by PAPPENHEIMER *et al* (1951) for the penetration from blood to the interstitial tissue. According to this view the fairly close correlation noted earlier between "clearance factors" and the free diffusion coefficients, in spite of the varying restriction put upon diffusion, is due to compensation (and overcompensation) by a constant contribution of solvent drag.

Even if the net absorption of water from the injection zone proceeds slowly the bulk flow may be considerable by way of the paracapillary circulation and lymph flow

Accepting the same diffusion coefficients as previously and assuming that hyaluronidase always completely abolishes the restriction to diffusion a solvent drag corresponding to an increment in the diffusion coefficients of about 0 15 $\times 10^{-5}$ cm^2 sec^{-1} appears to give the best fit to both sets of clearance values. However the relatively few results, the experimental error involved and in particular uncertainties about the free diffusion coefficients of our inulin and dextran preparation (the values have been taken from the literature and were not determined for our particular heterogenous compounds), as also the possibility of histamine liberation

by dextran – all these indicate that a quantitative interpretation must be treated with reserve at the present time

The previous statement should be extended to the statement that diffusion is one of the main and may for the smaller molecules and ions be in fact the main driving force for their absorption. Since, however the hindrance to diffusion increases with the size of the diffusing compound, the transport by solvent drag is likely to be of greater importance the greater the molecule. For molecules as large as dextran solvent drag is probably the principal factor

There is a tendency for the effect of hyaluronidase on the smaller sized compound to decrease with time (table 2) Since hyaluronidase itself is a large molecule this effect could well be expected. MALMGREN (1953) estimated a.m.v of 11 000 for the testicular preparation

Dextran It has repeatedly been shown, for instance, by SEMPLE (1954) and GROTTE (1956) that even large molecular weight fractions of dextran are able to pass from blood to the tissues. This is substantiated in outflow experiments with our dextran preparation (fig. 3). The fairly large distribution volume found after 4 hours suggests that, under the experimental conditions of our muscle clearance experiments, dextran will cross most capillary walls, including the muscle capillaries.

The information about the exit of dextran was obtained in double tracer experiments with ^{3}H labelled inulin as a reference substance that is distributed in the extracellular compartment. The curves in fig. 3 shows that dextran is distributed at a significantly lower rate than inulin. It could be tempting to compare the exit rate for the two compounds according to the method described by KRUHOFFER (1946) We have refrained from attempting such a quantitative comparison, however because of the heterogenity of the dextran preparation and possible liberation of histamine by this compound factors that may alter the rate-determing pattern for the exit during a given time

The decline in the concentration of dextran in the blood might not be due solely to transcapillary passage, if the substance was taken up by macrophages at specific sites. In several of the dextran experiments we measured the radioactivity in the spleen, liver and bone marrow after processing. We did not find any accumulation of radioactivity in these tissues.

Moreover in recent experiments (unpublished), Schou was able to sample radioactive blood from the catheterized *vena cava inf* immediately after injection of radio-dextran into the thigh muscles of rats. Since the lymph drainage through the thoracic duct in these animals is only about 1 ml/hour (BOLLMAN *et al* 1948) the results clearly indicate that the absorption of dextran proceeds essentially across the capillary wall and not by lymphatic drainage.

Hyaluronidase did not split the polysaccharide chain of dextran. The experiments thus show that even the large-sized fractions of dextran are absorbed at a considerable rate when the enzyme is added Otherwise, the absorption of these fractions is thought to manifest itself by the rapid decrease in the clearance factor of dextran with time. Thus, in the absence of hyaluronidase, factors of 1.5 1.2, 0.33 and 0 13 were found for the four experimental periods, compared with 3.2, 1 9 1 4 and 1 1 in presence of the enzyme. This gives rise to an increase in the ratios of clearance factor (F_{H}/F_{O}) with time.

It seems difficult to explain the difference in behaviour entirely by an increased diffusion rate of dextran The compound is known to be a histamine liberator The time-course of any possible histamine liberation in the two conditions, and the relative amounts of histamine liberated, should perhaps be considered. Any increase in the pore size of the capillary wall caused by histamine could have a pronounced effect upon osmotic water flow hence upon solvent drag effect, as pointed out by USSING (1961).

Summary

Labelled mannitol, sucrose, inulin (m. w 3 000-4,000) and dextran (m. w 60.000-90.000) were injected with hyaluronidase (300 I.U /ml) in a small volume (16 µl) into the exposed *m extensor quadriceps femoris* of anaesthetized rats. The absorption rates obtained were inversly related to molecular size as estimated from the amounts absorbed during the first 5 min., they were less satisfactorily correlated with the free diffusion coefficients of the compounds. The enzyme enhanced absorption of each compound, but to a different degree During the first 5 min the mean increase in absorption rate compared with control values was for mannitol about 10 %, sucrose 20 % inulin 30 % and dextran about 100 % The clearance of ^{3}H-water was not enhanced by hyaluronidase

The experiments provide evidence that the extent of the resistance to diffusion provided by the intramuscular connective tissue depends on the molecular weight of the diffusing substance. Further a significant solvent drag is superimposed upon the diffusion rates, the absorption rates being determined by the combined effect.

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The Local Effect of Cholinergic Agents and Cholinesterase Inhibition on the Clearance of Sucrose from Muscles

By

Aksel F. Jørgensen and Jens Schou

(Received April 26, 1965)

It has recently been reported that the absorption rate of sucrose injected into rat muscles is decreased by the simultaneous local administration of atropine or any of several other anticholinergic drugs (SUND & SCHOU 1964b)

As a working hypothesis, these authors suggested a *cholinergic system* constantly liberating acetylcholine as of physiological importance in maintaining a sufficient nutritional capillary flow If the precapillary sphincters were constantly dilated by acetylcholine, the desensitization by anticholinergic agents could reduce the nutritional blood flow and thereby decrease the absorption rate for sucrose.

In his fundamental work on the ^{24}NaCl-clearance from muscles as measure of regional circulation, KETY (1948) showed that methacholine accelerated the clearance of radioactive sodium chloride. The intra arterial infusion of methacholine has further been shown to increase blood flow as well as clearance rate of $Na^{131}I$ from cat muscles (HYMAN *et al* 1959)

In our experiments the method for absorption studies described by SUND & SCHOU (1964a) was used. The rates of clearance of ^{14}C labelled sucrose, injected intramuscularly were determined from solutions con taining either one of the two cholinergic agents, acetylcholine and carba choline, or the cholinesterase inhibitor paraoxon (paroxan). Special attention was given to the effects of varying concentrations of the drugs and to the quantitative drug response at various times during the first 15 minutes after the injection.

Methods

Male albino rats of a single strain, weighing 110–140 g, were maintained on a standard laboratory diet with water ad libitum.

The absorption experiments were performed as described by SUND & SCHOU (1964a). Briefly 16 µl of a solution, containing labelled sucrose, the drug and sodium chloride to isotonicity were injected into the exposed *m. extensor quadriceps femoris* of the rats, anaesthetized with mebumal (pentobarbital) intraperitoneally. At various times after injection (5, 10 and 15 min.) the muscles were removed. After chemical treatment (SUND & SCHOU 1964a) the remainder of the injected sucrose was determined as radioactivity measured by liquid scintillation technique. The amount of sucrose remaining at the end of the experimental period was expressed as percentage of the amount injected.

Labelled sucrose

^{14}C-sucrose, uniformly labelled, with specific activity of 36.1 mC/g, was obtained from the Radiochemical Centre, Amersham, England.

Injection solutions

The three types of injection solutions were all prepared in glass-distilled water containing 0.9 sodium chloride and radiosucrose at tracer concentration (0.35 mg/ml, corresponding to 12.6 mC/ml)

1. Acetylcholine iodide, unlabelled, was used at concentrations from 0.55 µM/l (0.15 µg/ml) up to 5500 µM/l (1.5 mg/ml)

2. Carbachline chloride, unlabelled, was used at concentrations from 0.27 µM/l (0.05 µg/ml) up to 2700 µM/l (0.5 mg/ml).

3. Paroxan (= paraoxon = diethyl-p-nitrophenyl phosphate) was used at concentrations from 8.4 µM/l (2.3 µg/ml) up to 2500 µM/l (0.7 mg/ml).

Results

Cholinergic agents

The percentage residual amounts of sucrose in the extensor quadriceps femoris muscle were measured 5 and 15 min. after injection of solutions containing 0.55, 5.5, 55, 550 and 5500 µM/l *acetylcholine iodide* (table 1). At 5 min. after the injection the amount remaining is significantly lower than in the controls in the experiments with 55, 550 and 5500 µM/l, demonstrating that an increased clearance rate for sucrose is effected by these concentrations. At 15 min., only the experiments with the two last mentioned concentrations of acetylcholine showed a significant *lowering* compared with control values.

To permit comparison of the effect of the drug on the rate of sucrose absorption in the two periods 0–5 and 5–15 min. the "clearance factor" (F) defined by SCHOU & SUND (1965), has been used.

This factor is defined as

Table 1

The effect of varying concentrations f *acetylcholine iodide* pon the bsorption rate of sucrose (0.35 mg/ml), I jected in 0.9 % saline into th *m. extensor quadriceps femoris* of the rat. The figures denot the percentage f the injected mount of sucrose ± s.e.m. remaining 5 d 15 minutes fter the injections. Figures in brackets indicate number of experiments.

Acetylcholine	Control					
Concentration (μM/l)	0	0.55	5.5	55	550	5500
Concentration (μg/ml)	0	0.15	1.5	15	150	1500
Dosage per injection (μg)	0	0.0024	0.024	0.24	2.4	24
5 min.	50.6 ± 1.8 (10)	53.8 ± 1.7 (8)	48.1 ± 2.1 (8)	40.0 ± 2.7[2] (8)	34.4 ± 2.6[3] (7)	28.7 ± 2.5[3] (8)
15 min.	20.3 ± 1.8 (8)	–	21.8 ± 3.1 (6)	17.5 ± 1.9 (6)	14.1 ± 1.8[1] (6)	9.1 ± 1.4[2] (8)

1) $P < 0.05$ 2) $P < 0.01$ 3) $P < 0.001$

$$F = \frac{(R_1 - R_2)\ 100}{R_1} \quad \frac{1}{t_2 - t_1}$$

where R_1 = residual amount at time t_1 and
R_2 = – – – – t_2 ($t_2 > t_1$).

Mathematically the factor expresses the average absorption rate (%/min) in the period ($t_1 - t_2$) when the amount existing at the beginning of the period is taken as 100 %.

In table 4 (part 1) is shown the ratio F_a/F_c of the clearance factors for the concentrations of acetylcholine applied (F_a) and the control experiments (F) in the two periods. This ratio expresses the extent to which the absorption process in the experimental condition (with acetylcholine) is accelerated compared with the control. The figures in table 4 confirm the accelerating effect of acetylcholine at concentrations of 55 to 550 μM/l on the absorption rate of sucrose in the period 0–5 min. During the period 5–15 min. an increased absorption rate was found only in the experiment with an initial concentration of 5500 μM/l acetylcholine. The apparent depression of the absorption rate obtained with 0.55 μM/l in the period 0–5 min., and in the period 5–15 min for the experiments with an initial concentration of 5 5 and 55 μM/l acetylcholine, cannot be considered significant, although it may be of relevance (see below).

With *carbacholine chloride* significantly lower residual amounts of sucrose were found in the muscles after 5 min. when the drug was added in concentrations from 27 to 540 μM/l (table 2). Higher and lower concentrations than these did not significantly affect the residual amounts after 5 minutes. With the higher concentrations, (1350 and 2700 μM/l), the animals were systematically affected by carbacholine, showing signs of general (absorptive) vascular insufficiency In a single experiment the absorptive effect of carbacholine was partly inhibited by the intraperitoneal injection of 28 μg atropine sulphate 3 minutes before the intramuscular injection of sucrose containing 2700 μM/l carbacholine. Five minutes later a residual 38 9 ± 2.5 % of the injected sucrose was found in the muscles, compared to 50 4 ± 1.5 % in a control experiment involving intraperitoneal atropine injection without carbacholine in the local sucrose solution. At 10 and 15 min. after the injections, significantly lower residual amounts of sucrose were found in the experiments with carbacholine at concentrations from 2.7 to 540 μM/l (table 2).

These results have been used for calculating the ratios $F_{carbacholine}/F_{controls}$ shown in table 4 (part 2) In the period 0–5 min., the accelerating effect of carbacholine at concentrations of 27 to 540 μM/l is again appearant. During the period 5–15 min even initial concentrations down to

Table 2

The effect of varying concentrations of *carbacholine chloride* upon the absorption rate of sucrose (0.35 mg/ml) injected in 0.9% saline into the *m. extensor quadriceps femoris* of the rat. The figures denote the percentage of the injected amount of sucrose ± s.e.m. remaining 5, 10 and 15 minutes after the injections. Figures in brackets indicate number of experiments.

Carbacholine	Control								
Concentration (µM/l)	0	0.27	2.7	27	135	270	540	1350	2700
Concentration (µg/ml)	0	0.05	0.5	5	25	50	100	250	500
Dosage per injection (µg)	0	0.0008	0.008	0.08	0.4	0.8	1.6	4	8
5 min.	50.6 ± 1.8 (10)	53.8 ± 4.1 (8)	53.1 ± 2.0 (8)	43.3 ± 2.4[1] (8)	33.5 ± 1.2[2] (6)	32.2 ± 2.3[3] (6)	29.1 ± 1.5[3] (6)	48.2 ± 1.5 (10)	50.0 ± 1.9 (11)
10 min.	32.1 ± 2.2 (8)	26.5 ± 1.9 (6)	18.4 ± 2.0[3] (6)	19.1 ± 1.4[3] (6)	–	16.2 ± 3.5[3] (6)	13.0 ± 2.3[3] (6)	–	–
15 min.	20.3 ± 1.8 (8)	16.9 ± 2.1 (6)	10.0 ± 1.2[2] (6)	9.5 ± 1.1[3] (6)	–	8.6 ± 0.9[3] (6)	5.2 ± 1.2[2] (6)	–	–

[1] $P < 0.05$ [2] $P < 0.01$ [3] $P < 0.001$

0.27 µM/l seemed to accelerate absorption. The lowering of the 0–5 min. ratios by 0.27 and 2.7 µM/l is not significant (compare table 2).

Cholinesterase inhibitor

With *paroxan* (paraoxon) in the injected solutions, the residual sucrose after 5 min was significantly decreased for the concentrations 840 and 2500 µM/l (table 3). At 15 min also 84 µM paroxan decreased the residual amount of sucrose (at the 5 % limit of probability). The effect of the cholinesterase inhibitor also is shown by the figures in table 4 part 3.

Discussion

The absorption rate of sucrose from muscles was found to increase gradually when the cholinergic agents acetylcholine (iodide) and carbacholine (chloride) were added to the injected solutions at increasing concentrations above certain thresholds (ca. 55 and 2.7 µM/l for the two drugs, respectively).

Sucrose was used in tracer concentrations only with sodium chloride added to isotonicity. It is pharmacodynamically inactive and represents a substance for which absorption is mainly due to diffusion through water filled pores that penetrate the capillary wall (conf. SUND & SCHOU 1964a, SCHOU & SUND 1965).

The effect of cholinergic agents upon sucrose clearance is most likely due to an increase in local nutritional blood flow caused by a dilatation of precapillary sphincters. An increase in the number of active capillaries in the absorption area lowers the mean distance required for sucrose

Table 3

The effect of varying concentrations of *paroxan* (= paraoxon, a cholinesterase-inhibitor) upon the absorption rate of sucrose (0.35 mg/ml) injected in 0.9 % saline into the *m. extensor quadriceps femoris* of the rat. The figures denote the percentage of the injected amount of sucrose ± s.e.m. remaining 5 and 15 minutes after the injections. Figures in brackets indicate number of experiments.

Paroxan	Control				
Concentration (µM/l)	0	8.4	84	840	2500
Concentration (µg/ml)	0	2.3	23	230	700
Dosage per injection (µg)	0	0.037	0.37	3.7	11.2
5 min.	50.6 ± 1.8	53.8 ± 4.2	45.6 ± 4.5	35.2 ± [illegible] [3]	31.6 ± [illegible] [3]
	(10)	(6)	(6)	(6)	(6)
15 min.	20.3 ± 1.8	19.3 ± 4.4	14.5 ± 1.8 [1]	12.4 ± [illegible] [3]	7.4 ± [illegible] [3]
	(8)	(6)	(6)	(6)	(6)

1) $P < 0.05$ 2) $P < 0.01$ 3) $P < 0.001$

Table 4

Ratios of clearance factors

Ratios of clearance factors (cf. text) for the absorption periods 0–5 min. and 5–15 min., with various concentrations of acetylcholine, carbacholine or paroxan in the ^{14}C-sucrose solution. The ratio (F_e/F_a) denotes the increase in the absorption rate caused by the drugs.

Acetylcholine concentration (µM/l)	0.55	5.5	55	550	5500	
0– 5 min.	0.93	1.1	1.2	1.3	1.4	
5–15 min.	–	0.92	0.93	1.0	1.1	
Carbacholine concentration (µM/l)	0.27	2.7	27	270	540	2700
0– 5 min.	0.93	0.95	1.1	1.4	1.4	1.0
5–15 min.	1.2	1.4	1.3	1.2	1.4	–
Paroxan concentration (µM/l)	8.4	84	840	2500		
0– 5 min.	0.93	1.1	1.3	1.4		
5–15 min.	1.1	1.1	1.1	1.3		

molecules to travel to a capillary wall and represents an increase in the total area of the absorbing membrane. Further the increase in capillary perfusion through the area allows a greater quantity of sucrose to be transported away per unit time. These factors provide the most probable and reasonable explanation for the increase in absorption rate. A dilatation of the waterfilled pores would increase the relative area of the capillary walls accessible for the diffusion of sucrose. This possibility is mentioned for completeness sake, although it is considered less probable that cholinergic agents would produce such an effect.

The accelerating effect of the cholinesterase inhibitor paroxan on sucrose absorption indicates endogenous liberation of acetylcholine, which reaches a concentration sufficient to allow an increase in nutritional blood flow during the action of the inhibitor. In previous experiments, atropine was shown to decrease the absorption rate of sucrose (SUND & SCHOU 1964b). The hypothesis that acetylcholine locally might be of importance for maintaining the actual level of capillary flow was suggested. This now seems more probable from the experiments with the esterase inhibitor described here.

For all three substances, concentrations below the threshold level were found to decrease the absorption rate of sucrose, although none of the single values differed significantly from the controls (tables 1, 2, 3 and 4). A similar reversal effect has been demonstrated for subactive atropine concentrations (SUND & SCHOU 1964b). Although only less importance should be devoted to these results, it might point to a difference between the sensitivity of the true precapillary sphincters on the one hand, and the

sphincters determining the flow through arterio-venous shunts on the other to the effect of physiological and pharmacological stimulation with vasoactive compounds

Summary

Radiosucrose (^{14}C) along with acetylcholine iodide, carbacholine chloride or the cholinesterase inhibitor paroxan (paraoxon) was injected into the exposed *m extensor quadriceps femoris* of anaesthetized rats in a constant volume Clearance rates of sucrose were studied by liquid scintillation counting.

Both the cholinergic agents and the cholinesterase inhibitor above certain threshold concentrations increased sucrose absorption with a positive dosage response.

The results indicate that acetylcholine locally plays a role in maintaining nutritional capillary flow

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The Influence of Thyroxine Treatment and Thyroidectomy on the Glycogenolytic and Lactic Acid Producing Effects of Adrenaline in Isolated Rat Diaphragm

By

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In experiments on fasted intact rabbits, it was found that thyroxine at a dose that raised the basal oxygen consumption by about 50 / increased the calorigenic effect of adrenaline and its capacity for raising the lactic acid content of the blood. Thyroidectomy reduced both these adrenaline effects (SVEDMYR 1965a). CORI (1931) suggested that the stimulating effect of adrenaline on lactic acid production in the blood was provoked by increased glycogenolysis in the skeletal muscles. It was therefore considered of interest to attempt to determine whether thyroid hormone influenced the glycogenolytic effect of adrenaline on striated muscle. A study was thus made of the way in which thyroxine and thyroidectomy affected the glycogenolytic and lactic acid stimulating effects of adrenaline in isolated diaphragms from fasted rats. In this tissue RIESSER (1947) WALAAS & WALAAS (1950) and WALAAS (1955) had previously demonstrated and studied the glycogenolytic and lactic acid-stimulating effects of adrenaline.

Method

Thyroxine treatment and thyroidectomy

The O_2-consumptions of the thyroxine-treated rats were determined both before treatment and after daily subcutaneous injections of 100 µg l-thyroxine (levaxin ®, Nyegaard) for 7 days. The weights of the animals ranged from 140 to 260 g. Thyroidectomy was performed 3–4 months before animals were used for the experiments. In order to prevent tetany 1 CaCl₂ was added to their drinking water for 7 days post-operatively. The O_2 consumptions were determined both before the operation and immediately before the animal was used. The weights of these animals ranged from 190 to 340 g.

The O_2 consumption was determined simultaneously in group of 4 animals by method previously described (SVEDMYR 1965a).

Preparation of the diaphragms

The animals were first fasted for 17–24 hours, in order that the preparatory treatment should be the same as in the rabbit experiments (SVEDMYR 1965a, b). They were then killed by a blow on the neck. The diaphragms from 3–4 animals were dissected out and immersed in ice-cold Krebs-Henseleit bicarbonate buffer without glucose. Bicarbonate buffer was chosen because the glycogenolytic effect of adrenaline appears to be greater in this than in phosphate buffer (SVEDMYR 1965c). The rat diaphragm varies considerably in thickness in different parts, and it may be assumed that its metabolism may also vary. In order to reduce this cause of variation as much as possible, 3–4 diaphragms, each of which was divided into two equal parts, were used for the experiments. The diaphragm halves used for the control and the adrenaline experiments were chosen in such a way that the two experimental groups were as similar as possible in their localization in the diaphragm.

The diaphragm halves were incubated at +2° in bicarbonate buffer without glucose for 30 minutes. This was done so that the initially high lactic acid content in the muscle would have time to decrease and become stable at a low value and so that the glycogenolysis and lactic acid production should reach a steady state. After this pre-incubation period the diaphragms in both control and adrenaline groups were once more divided. One of these fourths (basal preparation) from each divided diaphragm was dried rapidly with filter paper and frozen at −80° with difluordichlormethane (freon 12) containing dry ice. One third of each of these basal preparations was then taken for glycogen analysis. The remainder was homogenized with 5 ml 10% trichloracetic acid for lactic acid analysis. These analyses gave the basal values of glycogen and lactic acid in the combined pieces of diaphragm. The diaphragm samples in the control group were then incubated with 15 ml fresh bicarbonate buffer for 15 minutes at 37°. The adrenaline group samples were incubated in the same way but with the addition of adrenaline at a concentration of 10^{-4}. At the end of the incubation period ⅓ of each piece of muscle was taken for glycogen analysis and ⅔ for lactic acid analysis. The lactic acid content of the suspension solution was also determined.

The relatively high concentration of adrenaline was chosen for three reasons. In experiments on rabbits (SVEDMYR 1965a) thyroxine potentiated the lactic acid stimulating effect of adrenaline in high but not in low doses. The adrenaline concentration chosen was that found by WALAAS (1955) to have the maximal glycogenolytic effect in experiments on rat diaphragm. During the incubation period it is probable that part of the adrenaline is oxidized in the relatively alkaline oxygenated suspension solution and that the tissue is affected by a progressively decreasing adrenaline concentration. Oxidation-inhibiting media (ascorbic acid, sodium bisulphite) might have been considered for stabilizing the adrenaline, but they may possibly have other side effects, and I therefore decided against using any of them.

The composition of the bicarbonate buffer in M/l was: NaCl 0.12; KCl 0.0047; $CaCl_2$ 0.0025; $MgSO_4$ 0.0012; KH_2PO_4 0.0012; $NaHCO_3$ 0.025. When aerated with 95% O_2 + 5% CO_2 the solution had a pH of 7.4.

Method of analysis

The glycogen content was determined by Pflüger's method in the modification of LUNDHOLM & MOHME LUNDHOLM (1957). Three or four samples of different diaphragms with a total weight of 0.1–0.15 g were used for the analysis. The total quantity of glycogen in the

preparation was 153 ± 70 mg. With the method used this quantity of glycogen was determined in known solutions with a recovery of 87 ± 5.6 %.

To determine the lactic acid content of the muscle, the frozen pieces of diaphragm with total weight of 0.25–0.35 g were immersed in 5 ml ice-cold 10 % trichloracetic acid and homogenized in an Ultra-Turrax apparatus. The homogenate was centrifuged and the lactic acid content of the extracts and incubation solutions was, I most experiments, determined by the method of FRIEDMANN & GRAESER (1933). As it was found, in some experiments, that lactic acid formation exceeded that of glycogenolysis the result was, in some experiments, checked with a more specific enzymatic method (LUNDHOLM, MOHME-LUNDHOLM & VAMOS, 1963). This control confirmed the results btained with the other method. The accuracy of the method has been tested in a previous study (LUNDHOLM, MOHME-LUNDHOLM & SVEDMYR, 1963).

The mount of glycogenolysis was determined from the difference in glycogen content between the basal preparation at the beginning and the control or adrenaline preparation at the end of the incubation period. The lactic acid production was determined from the difference between the sum of the lactic acid contents of the muscle and suspension solution at the end of the experiment and the lactic acid content at the beginning of the experiment.

Results

Oxygen consumption in thyroxine-treated and thyroidectomized rats

Thyroxine at a dose of 100 µg/animal/day for 7 days increased the oxygen consumption by an average of 30 % of the basal value Thyroidectomy decreased the consumption by an average of 25 %.

The basal glycogen content of the muscle was of significance it constituted an upper limit for the degree of glycogenolysis and lactic acid production, expecially since the experiments were carried out on fasted animals. As mentioned above, no glucose was present in the suspension medium. The basal glycogen contents are therefore given below The amount of spontaneous glycogenolysis and lactic acid production in the control preparations are also given. The effect of adrenaline on glycogenolysis and lactic acid production is reported in two ways, first as the total effect and secondly as the change in relation to spontaneous glycogenolysis and lactic acid production, named below the "adrenaline effect

Basal glycogen content The basal glycogen content was highest in the diaphragms from thyroidectomized animals (174 8 ± 28 0 mg / the mean of 5 experiments on 14 animals) and lowest in the thyroxine-treated (120 9 ± 15.2 mg / 13 experiments on 45 animals) In the normal animals the content was 149 1 ± 13 7 mg (17 experiments on 51 animals) In none were the differences significant. It cannot therefore be assumed with certainty that variations in the basal glycogen content contributed to the differences in glycogenolysis and lactic acid production in the different groups.

Spontaneous glycogenolysis As shown in table 1 a certain degree of spontaneous glycogenolysis occurred in the control experiments on the

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The glycogen content was determined by Pflüger' method in the modification of LUNDHOLM & MOHME LUNDHOLM (1957). Three or four samples of different diaphragms with a total weight of 0.1–0.15 g were used for the analysis. The total quantity of glycogen in the

preparation was 153 ± 70 mg. With the method used this quantity of glycogen was determined in known solutions with recovery of 87 ± 5.6 %.

To determine the lactic acid content of the muscle, the frozen pieces of diaphragm with a total weight of 0.25–0.35 g were immersed in 5 ml ice-cold 10 % trichloracetic acid and homogenized in Ultra-Turrax apparatus. The homogenate was centrifuged and the lactic acid content of the extracts and incubation solutions was, in most experiments, determined by the method of FRIEDEMANN & GRAESER (1933). As it was found, in some experiments, that lactic acid formation exceeded that of glycogenolysis the result was, in some experiments, checked with a more specific enzymatic method (LUNDHOLM, MOHME-LUNDHOLM & VAMOS, 1963). This control confirmed the results obtained with the other method. The accuracy of the method has been tested in a previous study (LUNDHOLM, MOHME-LUNDHOLM & SVEDMYR 1963).

The amount of glycogenolysis was determined from the difference in glycogen content between the basal preparation at the beginning and the control or adrenaline preparation at the end of the incubation period. The lactic acid production was determined from the difference between the sum of the lactic acid contents of the muscle and suspension solution at the end of the experiment and the lactic acid content at the beginning of the experiment.

Results

Oxygen consumption in thyroxine-treated and thyroidectomized rats

Thyroxine at a dose of 100 μg/animal/day for 7 days increased the oxygen consumption by an average of 30 % of the basal value. Thyroidectomy decreased the consumption by an average of 25 %.

The basal glycogen content of the muscle was of significance; it constituted an upper limit for the degree of glycogenolysis and lactic acid production, expecially since the experiments were carried out on fasted animals. As mentioned above, no glucose was present in the suspension medium. The basal glycogen contents are therefore given below. The amount of spontaneous glycogenolysis and lactic acid production in the control preparations are also given. The effect of adrenaline on glycogenolysis and lactic acid production is reported in two ways, first as the total effect and secondly as the change in relation to spontaneous glycogenolysis and lactic acid production, named below the "adrenaline effect".

Basal glycogen content. The basal glycogen content was highest in the diaphragms from thyroidectomized animals (174.8 ± 28.0 mg %, the mean of 5 experiments on 14 animals) and lowest in the thyroxine treated (120.9 ± 15.2 mg %, 13 experiments on 45 animals). In the normal animals the content was 149.1 ± 13.7 mg (17 experiments on 51 animals). In none were the differences significant. It cannot therefore be assumed with certainty that variations in the basal glycogen content contributed to the differences in glycogenolysis and lactic acid production in the different groups.

Spontaneous glycogenolysis. As shown in table 1, a certain degree of spontaneous glycogenolysis occurred in the control experiments on the

Preparation of the diaphragms

The animals were first fasted for 17 24 hours, in order that the preparatory treatment should be the same as in the rabbit experiments (SVEDMYR 1965a, b). They were then killed by a blow on the neck. The diaphragms from 3–4 animals were dissected out and immersed in ice-cold Krebs Henseleit bicarbonate buffer without glucose. Bicarbonate buffer was chosen because the glycogenolytic effect of adrenaline appears to be greater in this than in phosphate buffer (SVEDMYR 1965c). The rat diaphragm varies considerably in thickness in different parts, and it may be assumed that its metabolism may also vary. In order to reduce this cause of variation as much as possible 3–4 diaphragms, each of which was divided into two equal parts, were used for the experiments. The diaphragm halves used for the control and the adrenaline experiments were chosen in such a way that the two experimental groups were as similar as possible in their localization in the diaphragm.

The diaphragm halves were incubated at +2 in bicarbonate buffer without glucose for 30 minutes. This was done so that the initially high lactic acid content in the muscle would have time to decrease and become stable at low value and so that the glycogenolysis and lactic acid production should reach a steady state. After this pre-incubation period the diaphragms in both control and adrenaline groups were once more divided. One of these fourths (basal preparation) from each divided diaphragm was dried rapidly with filter paper and frozen at −80 with difluordichlormethane (freon 12) containing dry ice. One third of each of these basal preparations was then taken for glycogen analysis. The remainder was homogenized with 5 ml 10% trichloracetic acid for lactic acid analysis. These analyses gave the basal values of glycogen and lactic acid in the combined pieces of diaphragm. The diaphragm samples in the control group were then incubated with 15 ml fresh bicarbonate buffer for 15 mi utes t 37 The drenaline group samples re incubated in the same way but with the addition of adrenaline at a concentration of 10^{-6} At the end of the incubation period ⅓ of each piece of muscle was taken for glycogen analysis and ⅔ for lactic acid analysis. The lactic acid content of the suspension solution was also determined

The relatively high concentration of adrenaline was chosen for three reasons. In experiments on rabbits (SVEDMYR 1965) thyroxine potentiated the lactic acid stimulating effect of adrenaline in high but not in low doses. The adrenaline concentration chosen was that found by WALAAS (1955) to hav the maximal glycogenolytic effect in experiments on rat diaphragm. During the incubation period it is probable that part of the adrenaline is oxidized in the relatively alkaline oxygenated suspension solution and that the tissue is affected by progressively decreasi g adrenaline concentration. Oxidation-inhibiting media (ascorbic acid sodium bisulphit) might have been considered for stabilizing the adrenaline but they may possibly have other side effects, and I therefore decided against using a y of them.

The composition of the bicarbonate buffer in M/l was NaCl 0.12 KCl 0.0047 CaCl 0.0025 MgSO 0.0012 KH_2PO_4 0.0012 $NaHCO_3$:0.025. When aerated with 95 O_2 + 5 CO_2 the solution had a pH of 7.4

Method of analysis

The glycogen content was determined by Pflüger method in the modification of LUNDHOLM & MOHME LUNDHOLM (1957). Three or four samples of different diaphragms with a total weight of 0.1–0.15 g were used for the analysis. The total quantity of glycogen in the

preparation was 153 ± 70 mg. With the method used this quantity of glycogen was determined in known solutions with recovery of 87 ± 5 6 %.

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Spontaneous glycogenolysis As shown in table 1 a certain degree of spontaneous glycogenolysis occurred in the control experiments on the

Table 1

Decrease in glycogen content in diaphragms from untreated, thyroxine treated and thyroidectomized animals. Spontaneous glycogenolysis and the effect of adrenaline. Glycogenolysis i mg/100 g wet muscle/15 min. n = number f tests. P = probability that the effect was due to chance.

		Control	Adrenaline	Change after adrenaline mean in the same exp.
Thyroidectomized	n = 5	-36.9 ± 19.3	-53.4 ± 18.0 $P < 0.05$	-16.5 ± 14.3
Untreated	n = 17	$+2.6 \pm 9.4$	-48.2 ± 8.6 $P < 0.001$	-50.4 ± 11.1 $P < 0.001$
Thyroxine-treated	n = 13	-12.4 ± 5.2 $P < 0.05$	-54.4 ± 9.4 $P < 0.001$	-42.5 ± 8.8 $P < 0.001$

	Difference between control values	Difference between adrenaline values	Difference between change after adrenaline
Untreated – thyroidectomized	39.4 ± 21.5	5.2 ± 20.0	33.9 ± 18.0
Untreated – thyroxine-treated	15.0 ± 10.8	6.2 ± 13.0	8.9 ± 14.2
Thyroxine-treated thyroidectomized	24.5 ± 20.0	1.0 ± 20.3	26.6 ± 16.6

thyroxine treated animals. A tendency to glycogenolysis (4 out of 5 experiments) was also observed in the control experiments on the thyroidectomized animals. No significant differences in the degree of spontaneous glycogenolysis were demonstrated between normal and treated animals.

Glycogenolysis after adrenaline After adrenaline significant glycogenolysis was shown in all three groups (table 1) in all, it was approximately 50 mg/100 g/15 min and no obvious differences between the total glycogenolysis in the different groups after adrenaline were observed. In the normal and thyroxine treated animals, however the adrenaline effect was significantly greater than spontaneous glycogenolysis The adrenaline effect in the thyroidectomized animals was significantly smaller than the sum of the effects in the normal and thyroxine-treated animals. The difference was 30.4 ± 15.0 ($P < 0.05$)

Spontaneous lactic acid production A significant degree of spontaneous lactic acid production was demonstrated in the control experiments in all groups (table 2)

Spontaneous lactic acid production was significantly higher in both the thyroxine-treated and the thyroidectomized group than in the untreated group

Table 2

Lactic acid production in diaphragms from untreated, thyroxine-treated and thyroidectomized animals. Spontaneous lactic acid production and the effect of adrenaline. Lactic acid production in mg/100 g/15 min. n and P as for table 1

		Control	Adrenaline	Change after adrenaline mean in the same exp.
Thyroidectomized	n = 5	+68.6 ± 17.0 $P < 0.02$	+65.2 ± 10.6 $P < 0.001$	−3.3 ± 10.0
Untreated	= 17	+ 4.1 ± 2.7 $P < 0.001$	+46.0 ± 5.6 $P < 0.001$	+ 1.9 ± 4.5 $P < 0.001$
Thyroxine-treated	n = 13	+38.3 ± 5.3 $P < 0.001$	+102.3 ± 9.5 $P < 0.001$	+64.0 ± 9.3 $P < 0.001$

	Difference between control values	Difference between adrenaline values	Difference between change after adrenaline
Untreated – thyroidectomized	−44.5 ± 17.2 $P < 0.02$	−19.8 ± 12.0	25.2 ± 11.0 $P < 0.05$
Untreated – thyroxine-treated	−14.2 ± 5.9 $P < 0.05$	−56.3 ± 11.0 $P < 0.001$	42.1 ± 10.6 $P < 0.001$
Thyroxine-treated thyroidectomized	30.3 ± 17.8	37.1 ± 14.2 $P < 0.02$	67.3 ± 13.8 $P < 0.001$

Lactic acid production after adrenaline Total lactic acid production after adrenaline was significantly greater in thyroxine treated animals than in the normal and thyroidectomized animals (table 2) In the normal and thyroxine-treated animals the adrenaline effect was significantly greater than spontaneous lactic acid production. On the other hand, no adrenaline effect was demonstrated in relation to the spontaneous lactic acid production in the thyroidectomized animals. In the thyroxine group the adrenaline effect was significantly greater than in the normal and the thyroidectomized groups. The adrenaline effect was also significantly greater in the normal group than in the thyroidectomized animals.

Discussion

Adrenaline stimulated both glycogenolysis and lactic acid production in diaphragms from normal and thyroxine-treated animals. On the other hand no unequivocal adrenaline effects were observed after thyroidectomy

Table 1

Decrease in glycogen content in diaphragms from untreated, thyroxine-treated and thyroidectomized animals. Spontaneous glycogenolysis and the effect of adrenaline. Glycogenolysis in mg/100 g wet muscle/15 min. n = number of tests. P = probability that the effect was due to chance.

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Glycogenolysis after adrenaline After adrenaline, significant glycogenolysis was shown in all three groups (table 1) In all, it was approximately 50 mg/100 g/15 min. and no obvious differences between the total glycogenolysis in the different groups after adrenaline were observed. In the normal and thyroxine treated animals, however the adrenaline effect was significantly greater than spontaneous glycogenolysis The adrenaline effect in the thyroidectomized animals was significantly smaller than the sum of the effects in the normal and thyroxine-treated animals. The difference was 30.4 ± 15.0 ($P < 0.05$)

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Discussion

Adrenaline stimulated both glycogenolysis and lactic acid production in diaphragms from normal and thyroxine-treated animals. On the other hand no unequivocal adrenaline effects were observed after thyroidectomy

Acknowledgements

I am indebted to Mr Theodor Dzedin for technical assistance. Financial support was provided by the Faculty of Medicine University of Gothenborg and the Swedish Society for Medical Research, Stockholm.

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The Effect of Thyroxine Treatment on Phosphorylase Activation by Adrenaline on Isolated Rat Diaphragm

By

Nils Svedmyr

(Received April 28 1965)

In previous experiments it was found that thyroxine increased the capacity of adrenaline to raise the lactic acid content of the blood in intact rabbits (SVEDMYR 1965a) In experiments on isolated diaphragms from thyroxine-treated and untreated rats (SVEDMYR 1965b) the stimulation of lactic acid production by adrenaline was found to be greatest in the former The glycogenolytic effect of adrenaline was not potentiated by thyroxine. There was a possibility however that a lack of glycogen had limited the glycogenolytic effect of adrenaline in these experiments.

The stimulation of carbohydrate metabolism by adrenaline in the skeletal muscles is assumed to be provoked by activation of the enzyme phosphorylase. Two forms of phosphorylase are found in muscle, *a* and *b* the *a* form is active in the absence of adenosine monophosphate (AMP), and the *b* form is only active in presence of a non-physiologically high concentration of AMP Adrenaline stimulates the transformation of phosphorylase *b* to phosphorylase *a* (RALL & SUTHERLAND 1960)

In considering the potentiating effect of thyroxine treatment on the stimulation of carbohydrate metabolism by adrenaline, it seemed of interest to determine whether thyroxine affected action of adrenaline on phosphorylase activation

Method

The rats to be treated with thyroxine were injected with 100 µg levoxin ® (Nygaard) per animal for 7 days This dose increased oxygen consumption by about 25%. The weights of both the thyroxine-treated and the untreated rats ranged from 250 to 350 g.

Each rat was killed by blow on the neck, and its diaphragm was dissected out od

divided into two equal parts, which were then weighed the weights of these halves ranged from 0.09 t 0.1. g. Each piece f diaphragm was incubated in a test tube containing 10 ml Krebs-Henseleit glucose-free bicarbonate buffer aerated with 5 CO_2 and 95% O_2, which produced a solutio of pH 7 40. After 30 minutes preincubation 10 µg of adrenaline dissolved in 0.1 ml of solution (0.9% NaCl + 0 05 Na_2SO_4 + HCl to pH 3.0 final drenaline concentration, 10^{-6}) were added to one sample 0.1 ml of the solution used for diluting the drenaline were added to the control sample. After 15 minutes incubatio each diaphragm sample was extracted in the cold with 4 ml of solution containing 0 02 N NaF + 0.001 M calcium tetracemin disodium (EDTA) by grinding them together with a little quartz sand in mortar After centrifugation 1 ml of the extract was diluted with 7 ml of a solution consisting of 0.04 M sodium glycerophosphate, 0 03 M cysteine hydrochloride and 0.2 M NaF whose pH was djusted to 6.8. Of this solution 0.8 ml was then taken for determining the phosphorylase activity as described by CORI, ILLINGWORTH & KELLER (1955). The activity was determined both in presence (0.001 M) and absence of AMP The reaction time was 15 minutes. The total activity is expressed as mg liberated P/g muscle/min. The whole of the incubation solution (1.6 ml) was analysed for inorganic phosphate as described by MARTIN & DOTY (1949). The phosphorylase activity is expressed as the amount of P liberated without AMP as a percentage of that liberated with AMP

Results

Table 1 shows that the phosphorylase *a* activity in the diaphragms from normal animals was 17 / and that the total phosphorylase activity corresponded to a liberated quantity of P of 0.24 ± 0 04 mg/g muscle/min. After adrenaline the phosphorylase *a* activity increased to 31 / and the total phosphorylase activity was also somewhat increased. After thyroxine treatment the total phosphorylase activity appeared somewhat higher than in the untreated animals, but the difference was not statistically significant. The phosphorylase-activating effect of adrenaline was not affected by thyroxine treatment.

Discussion

These experiments showed that the administration of thyroxine to normal animals did not affect phosphorylase activation by adrenaline in isolated rat diaphragm. These results are in concordance with previous experiments on the rat diaphragm (SVEDMYR 1965b) in which it was found that thyroxine treatment increased the stimulation of lactic acid produc tion by adrenaline but not its glycogenolytic effect. It has also been found that thyroxine treatment does not increase the enhancing effect of adrena line on the hexosephosphate content of the rat diaphragm (SVEDMYR, unpublished results) which indicates that the glycogenolytic effect of adrenaline is not affected by thyroxine. Thyroxine treatment alone affected neither the total phosphorylase activity nor the phosphorylase *a* activity of the

Table 1

The effect of adrenaline on the phosphorylase activity of diaphragms from untreated and thyroxine-treated rats. Total phosphorylase activity (T) was determined in the presence of 0.001 M AMP and is recorded as mg inorganic P liberated per g muscle per min. Phosphorylase activity is the amount of P liberated without AMP expressed as a percentage of that liberated with AMP. P = probability that the effect was due to chance; = number of tests.

	Untreated animals n = 10			Thyroxine-treated animals = 12		
	control	adrenaline	change after adrenaline	control	adrenaline	change after adrenaline
activity per cent	16.9 ± 2.[illegible]	31.1 ± 4.5	14.2 ± 2.3 $P < 0.001$	19.8 ± 3.7	34.7 ± 5.1	14.9 ± 3.0 $P < 0.01$
total activity P mg/g/min.	0.24 ± 0.04	0.32 ± 0.04	0.09 ± 0.018 $P < 0.001$	0.30 ± 0.04	0.38 ± 0.04	0.08 ± 0.02 $P < 0.01$

Difference in adrenaline effect o a activity between thyroxine-treated and untreated = +0.7 ± 1.8.
Difference in total phosphorylase activity in control tests between thyroxine-treated and untreated = 0.06 ± 0.06

diaphragm. This agrees with the report of HORNBROOK & BRODY (1963) that thyroxine treatment did not influence the phosphorylase activity of skeletal muscle from the rat.

Summary

It was demonstrated in previous experiments that the administration of thyroxine potentiated the stimulation of the carbohydrate metabolism by adrenaline both in the intact rabbit and in isolated rat diaphragm. It was found in the studies here recorded that the phosphorylase activation by adrenaline in isolated rat diaphragm was not affected by thyroxine given in a dose that increased the basal oxygen consumption by about 25 / in the treated animals. Neither did thyroxine increase total phosphorylase activity

Acknowledgements

I am indebted to Mr Theodor Dzedin for technical assistance. Financial support was provided by the Faculty of Medicine, University of Göteborg and the Swedish Society for Medical Research, Stockholm.

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Acta pharmacol et toxicol 1965 23 225–230

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The Effect of L-Thyroxine Treatment on the Metabolism of Tritium-Labelled Catecholamines in the Rat

By

Nils Svedmyr and Bertil Waldeck

(Received May 7 1965)

Thyroxine potentiates several of the effects of adrenaline and noradrenaline, both metabolic and cardiovascular (review Hoch 1962 Harrison 1964). Various explanations have been postulated One is that thyroxine influences the inactivation of the catecholamines (CA) in such a way that more amine is made available to the receptor mechanism. Another explanation is that the sensitivity of the receptors increases.

The aim of the investigation described here was to study the first of these two hypotheses.

The effects of a moderate dose of thyroxine on the concentrations of ^{3}H-noradrenaline (^{3}H NA), ^{3}H-adrenaline (^{3}H A) and the metabolites ^{3}H-normethanephrine (^{3}H NM) and ^{3}H-methanephrine (^{3}H M) were investigated in the cardiac muscle, femoral muscle, diaphragm and blood of the rat after infusion of these ^{3}H-CA. One series was performed with ^{3}H NA and one with ^{3}H A, since these two amines have different effects on the distribution of blood to different organs, especially the muscles, and also on a number of receptors. The thyroxine dose used has been found in other studies (Svedmyr 1965 a, b c, & d) to potentiate some of the adrenaline effects.

Material and Method

Thirty-three fully grown male rats with mean weight of 257 ± 5 g were used. In each series, simultaneous experiments were performed on group of rats given subcutaneous injections of 100 µg L-thyroxine per day for seven days, dissolved in isotonic sodium chloride solution adjusted t pH 11 by means f sodium hydroxide, and control group receiving the solvent alone. The oxygen consumption in the thyroxine-treated animals had increased by 30 % bove the basal level, but the body weight was unaffected.

In order to reduce the circulatory effect of the injected CA, this was given by intravenous infusion for 30 minutes in a total dose of 1 μg/kg (dl-form). The purity of the labelled CA was investigated by paper chromatography whereby one batch of ^{3}H A was found to contain at least 95 % of ^{3}H NA: such a check is thus obviously important.

When the oxygen consumption had been measured, the animals were placed in special cages (described by BERGLUND 1962) and a fine plastic catheter was introduced into one of the caudal veins by the method of BERGLUND (1962). The cages containing the rats were then placed in a separate cupboard, where they stood for the remainder of the experiment. The plastic tubes were drawn out through a hole in the cupboard door to an infusion pump, and 0.9 % sodium chloride solution with added heparin (total heparin dose 0.015 g) was infused at a rate of 0.1 ml/min. for 30 minutes in order to obtain basal conditions. The relevant ^{3}H-CA was then infused for 30 minutes at the same rate as the dosage mentioned above. When the infusion was completed, the animal was killed by a blow on the neck and then beheaded: the blood was collected, and a plasma extract was prepared by the method of HÄGGENDAL (1963). The heart, diaphragm and femoral muscle were quickly dissected out and extracted with perchloric acid by the method of BERTLER, CARLSSON & ROSENGREN (1958). Ion exchange chromatography was performed on the extract as described by CARLSSON & WALDECK (1963), the contents of ^{3}H NA and ^{3}H NM, and ^{3}H A and ^{3}H M being determined. Samples from animals that had been infused with ^{3}H A were eluted, however, as described below. The first 12 ml were discarded, the next 10 ml contained the ^{3}H A and the next 18 ml after contained the ^{3}H M. The ^{3}H M and ^{3}H NM determinations in the heart were excluded because of the lack of specificity (CARLSSON & WALDECK 1963). For further discussion on ion exchange chromatography see HÄGGENDAL (1963).

Results

The mean weight of all control animals was 257 g (s.e.m. = 9, n = 14), and the weight of the heart was 0.69 g (s.e.m. = 0.03, n = 14); the corresponding weights in the thyroxine-treated animals were 257 ± 6 (n = 19) and 0.80 ± 0.02 (n = 19), respectively. The thyroxine treatment had not altered the weight of the animals, but it did give rise to some cardiac hypertrophy, the difference in average heart weight from that of the control group being *0.11 ± 0.04 (P ≤ 0.01)*.

^{3}H A infusion (fig. 1)

The concentration of ^{3}H A in the heart was not significantly lower in the thyroxine-treated group. When correction was made for the cardiac muscular hypertrophy, the quantity of ^{3}H A was found to be exactly the same in both groups.

Striated muscle took up much less CA than cardiac muscle, probably because of its less abundant supply of adrenergic nerves (HERTING *et al.* 1961). Thyroxine treatment had no effect on the quantity of ^{3}H A taken up, and no significant difference between contents of ^{3}H M was obtained.

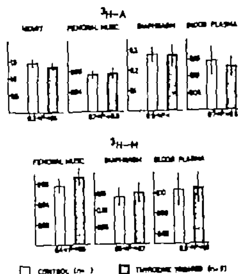


Fig. 1 The effect of thyroxine treatment on the turnover of infused tritium-labelled adrenaline in rat.
³H-A was infused in total dose of 1 μg/kg for 30 minutes. Thyroxine 0.10 μg/animal/day for 7 days. *Content of ³H A and ³H M in μg/g tissue immediately after the end of the infusion.*

The content of ³H A in the plasma appeared somewhat lower in the thyroxine-treated group but the difference again was not statistically significant. The contents of ³H M were also similar in the two groups.

³H NA infusion (fig. 2)

The results from animals infused with ³H NA were in the main the same as those for ³H A, but the scatter was somewhat greater the series of experiments having been spread out over a longer period

Discussion

Circulating CA is inactivated partly by the action of enzymes and partly by being taken up and bound in the tissues, where it is protected from breakdown for a certain period (AXELROD & TOMCHICK 1960 WHITBY AXELROD & WEIL MALHERBE 1961) Blocking of COMT especially in the liver (AXELROD *et al* 1958 AXELROD 1959 DE SCHAEPDRYVER & KIRSHNER 1961 CARLSSON & WALDECK 1963) and inhibition of the uptake of CA in adrenergic neurones (WHITBY HERTTING & AXELROD 1960) results in a higher concentration in the blood and at the receptors and in this way potentiates and prolongs its action if the receptors remain other

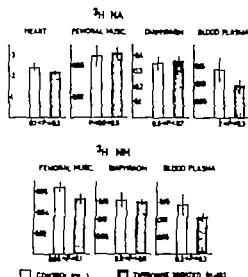


Fig. 2. The effect of thyroxine treatment on the turnover of infused tritium-labelled noradrenaline in the rat.

³H NA was infused in a total dose of 1 µg/kg f r 30 minutes. Thyroxine 0.10 µg/animal/day for 7 days. Content of ³H NA and ³H NM in µg/g tissue immediately fter the end of the infusion.

wise unchanged (WYLIE, ARCHER & ARNOLD 1960 CARLSSON & WALDECK 1963)

A number of investigations have been made on the concentration of CA in different organs both under normal conditions and after the administration of thyroxine (review HARRISON 1964). The results of these vary greatly possibly owing to the large differences between thyroxine doses used in the different investigations and also to the doses being often high. It is well known that with large doses of thyroxine a number of effects without physiological relevance can be obtained on different enzyme systems (review HOCH 1962) Large CA doses were used in several of these investigations.

BEAVAN, COSTA & BRODIE (1963) found in mice that a thyroxine dose that raised the oxygen consumption 75 / did not unequivocally affect the amount of endogenous noradrenaline (NA) in the heart. The content of tritium-labelled NA (³H NA) taken up was insignificantly reduced, and the ³H NA turnover in the cardiac muscle was not affected

WURTMAN, KOPIN & AXELROD (1963) however observed hypertrophy and a decreased ability of each unit weight of the hyperthyroid rat heart to inactivate ³H-adrenaline by binding.

In our investigation no significant effect of thyroxine treatment, in the dose used, was shown on the uptake of ³H NA or ³H A by the heart,

femoral muscle or diaphragm, nor was any effect on the ^{3}H NM or the ^{3}H-M content demonstrated

The concentration of ^{3}H-CA in the blood should rise after infusion of ^{3}H-CA if the uptake is reduced and if it is not metabolised to an increased degree. Inhibition of COMT as mentioned previously should also give an increased concentration in the blood and thereby increased uptake.

In our experiments the contents of both ^{3}H NA and ^{3}H A in the blood were the same in both series. The contents of ^{3}H NM and ^{3}H M were also unaltered, which indicates that in these experiments the uptake mechanism was not affected, and that thyroxine did not inhibit the COMT

It seems probable, therefore, that the sensitizing effect of thyroxine on the action of the injected catecholamines can to a minor degree only or not at all, be attributed to changes in CA uptake or breakdown but to a great extent depends on changes in the effector cells.

Summary

1 µg ^{3}H A or ^{3}H NA was infused intravenously for 30 minutes into fully grown male rats in two different series of experiments, each comprising a control group and a group receiving 0 10 µg L thyroxine per day subcutaneously for 7 days.

The oxygen consumption in the thyroxine treated animals had increased by 30 %. The uptakes of the labelled CA and of the metabolites ^{3}H NM and ^{3}H M were studied in the heart (^{3}H A and ^{3}H NA only) femoral muscle and diaphragm. No significant effects of thyroxine in the dose used were shown. The concentrations of ^{3}H NA and ^{3}H A, and ^{3}H NM and ^{3}H M, in the blood also remained unaffected by the thyroxine treatment. It is suggested that the sensitizing effect of thyroxine on catecholamines may be attributed to changes in the effector cells rather than to decreased uptake and metabolism.

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Excretion of Urinary Kinins in Rheumatoid Arthritis

By

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(Received May 17 1965)

In a previous paper (BRISEID JENSEN VENNERÖD & RINVIK 1965) values were given for the excretion of the urinary kinins Z_1 and Z_2 by healthy persons as well as by subjects suffering from various diseases. The kinin excretion in two cases of rheumatoid arthritis was significantly lower than in the control material, and an investigation of a larger number of such patients seemed desirable. Previous chromatographic and pharmacological experiments had suggested that bradykinin was the main constituent of Z_1 and kallidin of Z_2 (BRISEID JENSEN & VENNERÖD 1962a & b BRISEID JENSEN, VENNERÖD & DYRUD 1963 BRISEID JENSEN, RINVIK & VENNERÖD 1963)

In our work we have investigated the kinin excretion in 24-hour urine specimens from males with established rheumatoid arthritis. They were patients at a hospital for rheumatic diseases (Oslo Sanitetsforenings Revmatismesykehus, Head Erik Kåss, M.D)

Technique

The collection of urine specimens, preparation of concentrates of Z_1 and Z_2, and assay of the fractions was as previously described (BRISEID JENSEN, VENNERÖD & RINVIK 1965).

Results

The excretion of the kinin fractions Z_1 and Z_2 was estimated in 11 males, within the age range 44 to 66 years. The amounts of kinins excreted were calculated as bradykinin for Z_1 and kallidin for Z_2, and the results are given in table 1 as µg substance per 24 hours. The table shows that the average values of Z_1 and of Z_2 for the 11 patients were roughly the

same and that $Z_1/Z_2 = 0.8$ which was also found for the 12 healthy males previously examined (BRISEID JENSEN, VENNERÖD & RINVIK 1965) As proposed in the previous paper the Z_1 and Z_2 excretion values were generally calculated as average values from three 24-hour urine specimens for a period of 3–5 weeks.

Table 1 shows that the average total kinin excretion value was 23 4 µg. with a standard error of 2 6 The value was considerably higher than the average excretion value previously found for 2 patients suffering from rheumatoid arthritis (13.3 µg) but still significantly lower than that observed for 12 healthy males, which was 34.3 with a standard error of 3.5 (BRISEID JENSEN, VENNERÖD & RINVIK 1965) In the newer material 10 out of 11 subjects had a lower excretion of kinins than the average value for the control material.

Discussion

When considering the results, it should be borne in mind that no withdrawal of medical treatment took place during urine collection periods. Table 1 shows that 10 subjects were given salicylate alone or together with phenylbutazone, gold, chloroquine or prednisone. One patient received prednisone only If the test group and the control group previously considered might in other respects be considered equivalent, this would mean that the lower output of urinary kinins in the subjects with rheumatoid arthritis might be ascribed either to the therapy or to the pathological condition itself If the lower kinin excretion values are correlated with the disease, the fact might indicate a connection with the pathophysiology of the disease, or it might be only a secondary phenomenon

The decrease in excretion of urinary kinins might be caused by decreased formation (lower level of releasing enzymes or of kininogen) or by increased inactivation (higher level of kininase) The excretion might reflect alterations in the plasma kinin level or it might be due to local changes in the kidneys. Investigations in progress on the plasma kininogen and kininase activities in healthy persons and in subjects suffering from rheumatoid arthritis should provide more information on the subject.

Summary

The excretion of kinin fractions Z_1 and Z_2 in 24-hour urine specimens from 11 males with rheumatoid arthritis was investigated Previous chromatographic and pharmacological experiments had suggested that

Table 1

Urinary excretion of kinin fractions Z_1 and Z_2 in males with rheumatoid arthritis.

The kinin values refer to 24-hour urines.
The Z_1 values are expressed as µg bradykinin,
the Z_2 values as µg kallidin.
The fractions were tested on rat uterus.

Subject number	Treatment	Z_1		Z_2		$Z_1 + Z_2$
	Salicylate	8.4		8.8		
1	Phenylbutazone	12.4	9.4	11.3	9.8	19.[illegible]
		7.5		9.3		
		17.2		18.3		
2	Salicylate	11.2	12.7	14.8	13.2	25.9
		10.7		9.3		
		11.6		10.3		
	Salicylate	6.6		7.0		
3	Gold	13.5	11.2	21.0	14.8	26.0
		13.4		16.5		
	Salicylate	17.8		18.3		
4	Chloroquine	16.7	16.9	14.0	15.7	32.6
		16.1		14.8		
	Salicylate	5.8		7.0		
5	Prednisone	4.0	6.0	6.0	7.5	13.5
		8.2		9.5		
	Salicylate	12.4		17.3		
6	Gold	19.4	16.8	19.8	20.3	37.1
		18.7		23.8		
		11.7		17.3		
7	Salicylate	13.4	13.3	20.8	16.3	29.6
		14.8		10.8		
	Salicylate	4.8		8.0		
8	Gold	14.7	9.7	17.0	12.4	22.1
		9.6		12.3		
		7.3		8.0		
9	Prednisone	6.4	7.7	25.3	13.9	21.6
		9.3		8.3		
10	Salicylate	5.1	7.0	11.5	10.5	17.5
	Prednisone	8.9		9.5		
11	Salicylate	7.3	7.3	5.3	5.3	12.6
Averages			10.7		12.7	23.4(±2.6) s.e.m.

Z_1 consisted mainly of bradykinin, and that kallidin was the main constituent of Z_2

The average daily total kinin excretion, Z_1 calculated as bradykinin and Z_2 as kallidin, was 23 4 μg with a standard error of 2.6 The observed value was significantly (P = 0 05) lower than the excretion value previously found for 12 healthy males, 34.3 μg, with a standard error of 3.5

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Effects of Inhibitors on the In Vitro Inactivation of Bradykinin by Various Kininases

By

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(Received May 17 1965)

Several papers have been published on the effects of inhibitors on the *in vitro* inactivation of kinins by inactivating enzymes of different origin (WERLE & GRUNZ 1939 ARMSTRONG JEPSON, KEELE & STEWART 1955 LEWIS 1960 WERLE 1961 ERDÖS & SLOANE 1962 ERDÖS, RENFREW SLOANE & WOHLER 1963 AMUNDSEN & NUSTAD 1964 KRIVOY & KROEGER 1964 AMUNDSEN, WAALER, DEDICHEN LALAND LALAND & THORSDALEN 1964 and BRISEID JENSEN, RINVIK & VENNERÖD 1965) Some *in vivo* investigations have been carried out on the inhibition of plasma kininases (ERDÖS & WOHLER 1963 ERDÖS, RENFREW SLOANE & WOHLER 1963 and BRISEID JENSEN, DYRUD & RINVIK 1965).

In the work presented here some drugs used in the treatment of rheumatoid arthritis were tested for possible kininase-inhibiting effects. Previous experiments had shown a reduced output of urinary kinins in subjects with the disease mentioned (BRISEID JENSEN RINVIK & DYRUD 1965) In addition, the effects of some substances already known as kininase inhibitors were examined. This was done partly to test their activities under more standardized conditions than had been done previously and partly to see if it was possible to distinguish between the kininases examined by their behaviour against several inhibitors.

Technique

A. *Materials*

1 *Plasma substrate from human blood, with kininase* was prepared by the method of AMUNDSEN, NUSTAD & WAALER (1963), omitting the heating to 56 and the acidification to pH 2.

2. *Plasma substrate from rat blood, with kininase,* was prepared in the same way as the corresponding substrate from human blood.

3. *Erythrocyt kininase preparations* from human and rat blood were prepared as previously described (BRISEID JENSEN, RINVIK & VINDTRÅDO 1965).

4. *Rat small intestine kininase*. The small intestine of a 300 g male rat was cut, distally to the duodenum and proximally to the caecum, and washed thoroughly with 0.9 sodium chloride solution. The gut, which weighed about 6.5 g after carefully drying between filter papers, was cut into 1 cm pieces and homogenized with cooling by ice water for 10 minutes in 65 ml 0.9 % sodium chloride solution. The homogenate was centrifuged for 15 minutes at 2200 r p m. and 5 and the supernatant was distributed in 1 and 2 ml aliquots and kept at −20° until use.

5 *Chemicals*. *Bradykinin* (synthetic) in ampoules of 100 µg/ml, Sandoz, A.G., Basel, Switzerland. *Calcium chloride* Calciumchlorid Dihydrat krist. zur Analyse, Merck, A.G. Darmstadt, Germany *Carboxypeptidase B* (*CPB*), COB 6064 107 U/mg, Worthington Biochemical Corp. Freehold, New Jersey U.S.A. *Cobalt chloride* Kobalt (11)-chlorid zur Analyse, Merck, A.G Darmstadt, Germany *Disulfiram* Antabus ® D mex Chemical Division, Dumex Limited, Copenhagen, Denmark. *Ethylenediamine tetra-acetic acid disodium salt* (EDTA 2N) Titriplex 111 Merck, A.G Darmstadt, Germany *Hydrocortisone* "Leo" ® for application to the eye. 0.3 mg/ml in methylcellulose and water Leo Pharmaceutical Products, Copenhagen, Denmark. *Gold sodium thiomalate* Myocrisin ® in ampoules of 100 mg/ml, May & Baker Ltd., Dagenham, England.

Hydroxychloroquine sulphate Ercoquin ® A/S Farmaceutisk Industri, Oslo, Norway. *1 10-Phenanthroline* o-Phenanthroliniumchlorid, Merck, A.G Darmstadt, Germany *Sodium salicylate* N triumsalicylat, reinst, krystallisiert, Merck, A.G Darmstadt, Germany *Zinc chloride* Zinkchlorid, trocken zur Analyse, Merck, A.G Darmstadt, Germany

B Methods

For each kininase preparation the main steps of th experimental procedure were as described below

From preliminary experiments there was estimated the concentration of enzyme required to break down about 70–80 % of a standard concentration of bradykinin (1 µg/ml) in a standard time (8 minutes). The extent of inactivation was also observed after 16 nd 32 minutes, in order to secure that the enzyme preparation was active beyond the 8-minute incubation period.

Next, an nhibitor-effect curve was established by adding different amounts of inhibitor to the enzyme and substrate concentrations chosen, the other experimental conditions emaining unchanged. The concentration of inhibitor found to inhibit partly the inactivation, 20–50 f bradykinin being broken down after 8 minutes, was then used in a final experiment in which the inactivation was recorded at 8 16, and 32 minutes.

In few xperiments the substrate was preincubated with the *inhibitor before enzyme* was added

Th experiments with carboxypeptidase B, the erythrocyte kininases and rat intestine kininase were carried out in tris buffer (0.1 M pH 7.3), but no buffer was added in the plasma kininase experiments. Disulfiram was added dissolved in acetone, 0.05 ml per 1 10 ml (1.05–1 15) incubation mixture, the other chemicals and the kininase preparations in 0.9 % sodium chloride solution. Acetone was also added to the control solutions incubated in parallel. The incubations were carried out at 37

The same batches of kininase preparations were used for all the experiments.

Th inactivation experiments were stopped by adding 0 04 N hydrochloric acid to pH about 1.8 3.0 ml acid to bout 1 10 ml incubation mixture being needed (5 ml for the plasma kininase), and then heating for 10 minutes at 37 Each kininase preparation had

been tested in advance, to ensure that no enzyme activity was left after the acidification procedure. Just before assay 0.1 N sodium hydroxide was dded to pH 7.3 ± 0.1 about 1.3 ml being required per about 1 10 ml incubation mixture (about 1 9 ml for the plasma kininase experiments). The ctivities of the samples were tested o the isolated rat uterus as described previously (BRESSO JENSEN, RINVIK & VENNERØD 1965).

C. *Comments on the methods*

Comparatively high enzyme concentrations and correspondingly short incubation periods (8 minutes) were used in order to make less important the effect of contaminants and also the effect of time on inactivation of the enzymes. It should be noticed, however that this caused the reaction time f enzyme-inhibitor to be too short for a maximal effect. Experiments carried out with a preincubatio period of rat small intestine kininase and EDTA 2Na before adding the substrate showed a considerably increased inhibition (table 8).

The observations at 16 and 32 minutes demonstrated whether the enzyme in question was still active or not. If no kinin destruction took place after 8 or after 16 minutes, the enzyme might either be unspecifically inactivated (the highly purified CPB seemed to be inactivated fairly quickly at 37°) or the inhibitor had at that time reacted with the whole amount of enzyme. In that event lower concentration f inhibitor and a lo ger contact period between inhibito and enzyme (preincubation) would have been more appropriate.

The concentration of bradykinin chosen, 1.2 μg/ml, was of the same order as that previously found on enzymatic release in human plasma samples (BRESSO JENSEN RINVIK & VENNERØD 1965).

Hydrocortisone was chosen as the representative of the glucocorticoids used in rheumatoid arthritis because of its relatively high water-solubility and as physiologically occurring substance. Higher concentrations than 5.10^{-4} M could not be tested because of the low solubility

Disulfiram was tested to a limit of concentration of about 3.10^{-4} M, which just gave a turbid solution. At higher concentrations precipitate was formed.

The ethylenediamine tetra-acetic acid calcium disodium, zinc disodium and cobalt disodium chelates were prepared from equimolecular amounts of tetracemin-disodium and the chlorides of the metals mentioned. Sodium hydroxide was added to neutralize the hydrochloric acid formed.

Results

Concentrations of enzymes

Table 1 shows the results of experiments carried out to select suitable concentrations of the various kininases. The values quoted, do not, how ever apply to the disulfiram experiments. The acetone used to dissolve the substance increased the inhibition, and about 50/ higher kininase concentrations generally had to be used.

Relative effects of the inhibitors

Tables 2–6 show the results of the inhibitor kininase experiments. Seven potential inhibitors were tested for their effects on 5 kininase preparations of different origin. In table 7 the main values from tables 2–6 are summa

Table 1

Inactivation of bradykinin by kininases of different origins.
Dilutions of the enzyme preparations corresponding to inactivation of 70–80 of the substrate
Incubatio at pH 7.3 and 37
1.. μg bradykinin per ml incubation mixture.

Enzyme	Enzyme preparation per ml incubation mixture	% bradykinin after		
		8 min	16 min.	32 min.
Carboxypeptidase B	0.1 μg	20	11	3
Human plasma kininase	0 25 ml	23	2	1
Human erythrocyte kininase	0 01 ml	25	5	2
Rat erythrocyt kininase	0.005 ml	25	5	3
Rat small intestine kininase	0.1 ml	25	5	1

rized the concentrations of inhibitors that were approximately equiactive are given in molarities From the tables it can be seen that *phenanthroline* proved most active, concentrations of an order of 10^{-4} to 10^{-5} M being effective under the conditions chosen. Then followed *disulfiram* and *hydrocortisone* at concentration ranges of about $10^{-3} - 10^{-4}$ M. The gold preparation *myocrisin* ® *hydroxychloroquine sulphate* and *EDTA 2Na* were only active as inhibitors at rather high concentrations, about $10^{-2} - 10^{-3}$ M and *sodium salicylate* was inactive at a concentration of 3×10^{-2} M

It should be pointed out, however that such a comparison of different inhibitors presupposes that all the substances act at the same rate One must assume that the 8-minute contact time was too short for a maximal effect of the inhibitors, and that considerably lower concentrations would suffice with a longer contact period. It is also probable that this was more important for some of the inhibitors than for others, thus partly invalidat ing the comparison of effective dose ranges. The significance of contact time for the inhibitory effect of EDTA 2Na is evident from table 8 A 60-minute preincubation of the enzyme with inhibitor before adding bradykinin made a concentration of 1 mg/ml of the inhibitor far more active than 2 mg/ml without preincubation With no preincubation, 1 mg/ml of EDTA 2Na had no effect at all. Tables 2–6 show that the quantities of bradykinin recovered when there was no inhibition and also in the control experiments varied more for carboxypeptidase B than for the other enzymes This might be partly ascribed to the fact that carboxy peptidase B as a highly purified enzyme was particulary liable to destruc tion

Table 2

Inactivation of bradykinin by carboxypeptidase B in the presence of potential inhibitors.

The figures given refer to about 1.1 ml incubation mixture with 1.2 µg bradykinin and 0.1 µg enzyme.

Inhibitor	Incubat. time minutes	Inhibitor µg	Bradykinin %	Incubat. time minutes	Inhibitor µg	Bradykinin %
	8	0	26	8	0	19
Phenanthroline		50	49	–	100	55
	–	100	90	16	–	36
				3	–	24
	8	0	34			
Disulfiram	–	50	18			No inhibition at 200 µg
	–	100	34			
	–	200	13			
	8	0	34			
Hydrocortisone	–	45	43			No inhibition at 180 µg
		90	30			
		180	31			
	8	0	25			
Myocrisin ®	–	250	18			No inhibition at 1000 µg
	–	500	10			
	–	1000	17			
	8	0	28			
Hydroxychloroquine sulphate	–	1000	38			
		2000	36			
	–	4000	48			
	8	0	18	8	0	24
EDTA 2Na	–	2000	34		4000	50
		4000	66	16	–	47
				32	–	47
Sodium salicylate						No inhibition at 4800 µg

Table 3

Inactivation of bradykinin by human plasma kininase in the presence of potential inhibitors.

The figures given refer to bout 1 1 ml incubation mixt re with 1.2 μg bradykinin and 0.25 ml enzyme preparation.

Inhibito	Incubat. time minutes	Inhibitor μg	Brady-kinin /	Incubat. time minutes	Inhibito μg	Brady kinin
	8	0	33	8	0	33
Phenanthroline	–	12.5	58	–	[illegible]	67
	–	25	75	16	–	27
	–	50	84	32	–	3
	8	0	23	No inhibition		
Disulfiram	–	100	24	at 200 μg		
	–	200	4			
	8	0	[illegible]	8	0	25
Hydrocortisone	–	30	56		60	70
	–	60	70	16	–	42
	–	90	84	32	–	8
	8	0	25	8	0	25
Myocrisin ®	–	250	37		3000	58
	–	1000	46	16		7
	–	4000	58	32	–	2
	8	0	26	8	0	27
Hydroxychloroquine sulphate	–	1000	50		2000	65
	–	2000	57	16		79
	–	4000	91	32	–	2
	8	0	[illegible]	8	0	25
EDTA 2N		1000	56		2000	69
	–	2000	64	16		69
		4000	88	32		69
Sodium salicylate				No inhib tion at 4800		

Table 4

Inactivation of bradykinin by human erythrocyte kininase in the presence of potential inhibitors.

The figures given refer to about 1.1 ml incubation mixture with 1.2 µg bradykinin and 0.01 ml enzyme preparation.

Inhibitor	Incubat. time minutes	Inhibitor µg	Bradykinin %	Incubat. time minutes	Inhibitor µg	Bradykinin %
	8	0	26	8	0	26
Phenanthroline	–	2.5	55	–	5	69
	–	5	69	16	–	50
	–	10	104	3	–	37
	8	0	28	8	0	23
Disulfiram	–	5	47	–	50	55
	–	50	66	16		48
	–	100	67	32	–	48
				8	0	22
Hydrocortisone				–	150	55
				16	–	28
				32	–	15
	8	0	18	8	0	18
Myocrisin ®	–	250	44		1000	64
		500	55	16	–	52
	–	1000	64	32	–	52
	8	0	[illegible]	8	0	26
Hydroxychloroquine sulphate	–	1000	36	–	4000	60
		2000	47	16	–	43
		4000	60	32	–	32
	8	0	35	8	0	35
EDTA 2Na	–	1000	27		4000	56
	–	2000	35	16	–	43
		4000	55	32		37
Sodium salicylate				No inhibition t 4800 µg		

Table 5

Inactivation of bradykinin by rat erythrocyte kininase in the presence of potential inhibitors.

The figures given refer to about 1.1 ml incubation mixture with 1.2 µg bradykinin and 0.005 ml enzyme preparation.

Inhibitor	Incubat. time minutes	Inhibitor µg	Brady-kinin %	Incubat. time minutes	Inhibitor µg	Brady-kinin %
	8	0	29	8	0	29
Phenanthroline				–	1	71
	–	1	66	16	–	58
	–	5	82	3		22
	8	0	28	8	0	23
Disulfiram	–	25	42	–	50	60
	–	50	56	16	–	56
	–	100	70	32	–	47
				8	0	29
Hydrocortisone				–	150	45
				16	–	10
				32	–	7
	8	0	2[illegible]	8	0	17
Myocrisin ®	–	200	33		1000	60
	–	500	45	16	–	33
		1000	61	32		33
	8	0	21	8	0	23
Hydroxychloroquine sulphate		1000	36		4000	71
		2000	47	16		55
	–	4000	71	32		28
	8	0	[illegible]9	8	0	29
EDTA 2N	–	1000	37	–	3000	67
	–	2000	49	16		46
		4000	75	32	–	36
Sodium salicylate				No inhibition at 4000 µg		

Table 6

Inactivation of bradykinin by rat small intestine kininase in the presence of potential inhibitors.

The figures given refer to about 1.1 ml incubation mixture with 1.2 µg bradykinin and 0.1 ml enzyme preparation.

Inhibitor	Incubat. time minutes	Inhibitor µg	Bradykinin %	Incubat. time minutes	Inhibitor µg	Bradykinin %
Phenanthroline	8	0	[illegible]	8	0	22
	–	25	42	–	50	68
	–	50	66	16	–	57
		100	77	32	–	34
Disulfiram	8	0	19	No inhibition t 200		
	–	100	19			
	–	700	19			
Hydrocortisone				8	0	28
				–	150	56
				16		[illegible]
				32		4
Myocrisin ®	8	0	26	8	0	26
	–	250	28	–	750	56
		500	45	16	–	8
		1000	67	32	–	2
Hydroxychloroquine sulphate	8	0	32	8	0	22
	–	1000	59	–	4000	64
	–	2000	70	16	–	16
	–	4000	85	3	–	1
EDTA 2Na	8	0	24	8	0	24
		1000	24	–	4000	61
	–	2000	47	16	–	61
		4000	61	32		61
Sodium salicylate				N inhibition at 4800 µg		

Selective inhibition of the enzymes

Tables 2–7 show that only phenanthroline and EDTA 2Na inhibited all the kininase preparations, carboxypeptidase B being unaffected by the other inhibitors at the concentrations used Carboxypeptidase B was thus clearly distinguishable from the other bradykinin-inactivating enzymes examined Further human plasma kininase differed from the cellular enzymes inasmuch as disulfiram had no inhibiting effect on it at the highest possible concentration. Human and rat erythrocyte kininase seemed to be similar to each other almost the same concentrations of the two inhibitors being required for the same effects. The rat small intestine kininase required significantly higher concentrations of phenanthroline than did the erythrocyte kininases being further entirely unaffected by disulfiram it might accordingly be judged different from them. Even if it is considered that the enzyme preparation was highly impure, and that contaminants might react selectively with the two inhibitors mentioned, thereby increasing the amounts of them necessary our results seem to indicate a qualitative dissimilarity between the rat small intestine kininase on the one hand and the two erythrocyte kininases on the other

Inhibition of rat small intestine kininase by the calcium inc or cobalt chelate of EDTA 2Na

Table 8 shows that not only EDTA 2Na but also the calcium chelate, inhibited the inactivation of bradykinin by rat small intestine kininase. The inhibition, however was weaker than for the sodium salt, in spite of a 60-minute preincubation of inhibitor and enzyme The zinc chelate also inhibited the enzyme under the same conditions, but not the cobalt chelate.

Discussion

The selective inhibition by different inhibitors was used by ERDÖS, RENFREW SLOANE & WOHLER (1963) to distinguish between human plasma kininase and human erythrocyte kininase AMUNDSEN, WAALER, DEDICHEN, LALAND LALAND & THORSDALEN (1964) observed that urgocyton a material isolated from ox liver inhibited the kininases of human erythrocytes and of liver cell homogenates far more powerfully than it did the plasma kininase Besides some of the inhibitors used by the above mentioned authors, several other potential inhibitors of kininase activity were tested in our work Of the 5 enzyme preparations examined, the two erythrocyte preparations (human and rat) could not be distinguished from each other under the conditions chosen but carboxypeptidase B human plasma kininase and rat small intestine kininase were

Table 7

Inactivation of bradykinin by kininases of different origin in the presence of various potential inhibitors.
The concentrations of inhibitors giving approximately the same effect (inhibitor-time experiments) were taken from tables 2–6 and calculated as molarities.

Enzyme	Concentration (M) of inhibitor about						
	Phenanthroline	Disulfiram	Hydrocortisone	Myocrisin ®	Hydroxychloroquine sulphate	EDTA 2N	Sodium salicylate
Carboxypeptidase B	4 10^{-4}	No effect at 7 10^{-4}	No effect at 5 10^{-4}	No effect at 3 10^{-3}	No effect at 9 10^{-3}	10^{-2}	No effect at 3 10^{-2}
Human plasma kininase.	10^{-4}	No effect at 7 10^{-4}	2 10^{-4}	8 10^{-3}	4 10^{-3}	5 10^{-3}	No effect at 3 10^{-2}
Human erythrocyte kininase	2 10^{-4}	2 10^{-4}	4 10^{-4}	3 10^{-3}	9 10^{-3}	10^{-2}	No effect at 3 10^{-2}
Rat erythrocyte kininase	4 10^{-4}	2 10^{-4}	4 10^{-4}	3 10^{-3}	9 10^{-3}	8 10^{-3}	No effect at 3 10^{-2}
Rat small intestine kininase.	2 10^{-4}	No effect at 7 10^{-4}	4 10^{-4}	2 10^{-3}	9 10^{-3}	10^{-2}	No effect at 3 10^{-2}

Table 8

Inactivation of bradykinin by rat small intestine kininase in the presence of various potential inhibitors.

The figures given refer t about 1 1 ml incubation mixture with 1 2 μg bradykinin and 0.1 ml enzyme preparation.

Inhibitor	Time in minutes		Inhibitor	Bradykinin
	Preincub.	Incub	μg	
	0	8	0	34
	0	–	1000	34
EDTA 2Na	0	–	2000	51
	15	–	0	23
		–	2000	100
	15	8	0	-3
EDTA-Ca2Na	–	–	1250	38
	–	–	2500	42
	60	8	0	29
EDTA 2Na			1000	9
		16	–	81
		32	–	73
	60	8	0	1
EDTA-Ca.N	–		2600	62
	–	16		40
		32		17
	60	8	0	34
EDTA Zn2N			1250	63
			2500	63
	60	8	0	38
EDTA-C 2Na			1250	35
			2500	35

found to differ from the erythrocyte enzymes and from each other It must, however be borne in mind that the only purified enzyme preparation was the carboxypeptidase B and that contaminants in the other might mask similarities between the enzymes, different contaminants adsorbing to different extents the inhibitors used

It should be mentioned that EDTA 2Na was found to inhibit all the kininases examined and also carboxypeptidase B at high concentrations (0 01 M) FOLK, PIEZ, CARROLL & GLADNER (1960) did not find that EDTA affected carboxypeptidase B when tested at the same concentra

tion they did, however use hippuryl L-arginine as substrate. Further ERDÖS, RENFREW SLOANE & WOHLER (1963) could distinguish between human plasma kininase and human erythrocyte kininase by use of EDTA, which inhibited the former at 3×10^{-3} M but not the latter at 7×10^{-3} M. In our experiments the substance inhibited the plasma kininase at 5×10^{-3} M but also the erythrocyte enzyme at a higher concentration (10^{-2} M).

Some experiments were carried out with rat small intestine kininase and different chelates of EDTA. Both the Ca and the Zn-chelate were found to inhibit the enzyme, though to a less pronounced extent than did the EDTA 2Na, but the Co-chelate had no effect, in spite of a 60-minute preincubation time. The result might suggest Co to be a metal essential to the kininase in question, or it might mean that the Co-chelate would require a still longer chelate-enzyme contact time. ERDÖS, RENFREW SLOANE & WOHLER (1963) found that both the Ca and the Mg-chelates of EDTA inhibited the plasma kininase, but not the Co-derivate.

The concentrations of inhibitors necessary for inhibition were sometimes rather high. This might, however to some extent be attributed to the short incubation time used, 8 minutes. Experiments carried out with preincubation times of 15–60 minutes showed that the amount of inhibitor could then be considerably reduced. It is also probable that the different inhibitors require different times for development of maximal effect. Tables 2–6 show that EDTA 2Na will require a longer contact time than the other inhibitors, as the observed recoveries of bradykinin did not seem to be much reduced by extending the incubation time from 8 minutes to 16 and then to 32 minutes. This observation suggests that the concentrations of EDTA 2Na that had to be used to cause an inactivation of bradykinin of 20–50 / might suffice for complete inhibition of the actual amounts of enzymes. The stability of substrate in presence of EDTA however was not so pronounced for the two erythrocyte kininase preparations as for the other kininases. On the other hand, those two enzymes were strongly inhibited by another inhibitor disulfiram, even beyond the first 8 minutes incubation time.

Of the drugs tested because of their use in the treatment of rheumatoid arthritis hydrocortisone, gold sodium thiomalate (myocrisin ®) and hydroxychloroquine sulphate exhibited a more or less pronounced kininase-inhibiting effect, but sodium salicylate did not. EDTA 2Na, which was active at high concentrations only might also be incorporated amongst the rheumatoid arthritis drugs, being used experimentally (BOYLE, CLARKE, MOSHER & MCCANN 1961). The two enzymes of interest in this connection are human plasma kininase and human erythrocyte kininase. Both enzymes were inhibited by the 4 above mentioned inhibi-

tors, roughly the same concentration of inhibitors being required to inhibit the plasma enzyme and the erythrocyte enzyme.

The previous observation of a reduced excretion of kinins in urines from patients with rheumatoid arthritis (BRISEID JENSEN, RINVIK & DYRUD 1965) together with the present observations of inhibition of kininases by drugs used in the treatment of the disease, suggest a comparative investigation of kininogen and kininase activities in healthy persons as well as in patients with rheumatoid arthritis.

Summary

The effects of some potential kininase inhibitors (phenanthroline, disulfiram, hydrocortisone gold sodium thiomalate (myocrisin ®), hydroxychloroquine sulphate, EDTA-2Na and sodium salicylate) on 5 kininase preparations of different origin (carboxypeptidase B human plasma kininase, human erythrocyte kininase, rat erythrocyte kininase and rat intestine kininase) were tested

Of the inhibitors examined, only sodium salicylate showed no kininase-inhibitory effect. The combined use of several inhibitors made it possible to distinguish between carboxypeptidase B human plasma kininase, rat small intestine kininase and the two erythrocyte kininases. The erythrocyte kininases could not be distinguished from each other

Phenanthroline was the most effective inhibitor concentrations of the order of 10^{-4} to 10^{-5} M being required. Then came disulfiram and hydrocortisone at a concentration range of about 10^{-3} to 10^{-4} M Myocrisin ® hydroxychloroquine sulphate and EDTA 2Na were only active at fairly high concentrations, about 10^{-2} to 10^{-3} M The concentrations necessary may have been somewhat overestimated, since an incubation time of only 8 minutes was used In experiments carried out with EDTA 2Na and preincubation times of 15–60 minutes the concentration of inhibitor required was considerably reduced.

Besides EDTA 2Na the calcium and the zinc chelates inhibited rat small intestine kininase, but EDTA-Co2Na had no effect under the same conditions.

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Absorption of Mercury Vapour from the Respiratory Tract in Man

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It is well-known that inhalation of air contaminated with mercury vapour may lead to poisoning from the absorption of toxic amounts of mercury from the respiratory tract.

Extensive studies within the field of industrial hygiene, first performed by NEAL *et al* (1937 & 1941) have revealed a valid correlation between the mercury concentration in the atmosphere and the incidence of chronic mercury poisoning. On the basis of these studies, industrial hygenists in the U.S.A. have fixed a toxic threshold limit value for mercury vapour and inorganic mercury compounds in the atmosphere at 100 μg/m³ under conditions involving constant daily exposure within normal working hours. This threshold value is still maintained by the American Conference of Governmental Industrial Hygienists (ACGIH 1964). British investigators have advised a threshold limit value of 75 μg/m³ (BUCKELL *et al* 1946, BROOKS & HOLMES 1958). In Soviet Russia, the toxic threshold limit value for metallic mercury has been set at 10 μg/m³ and for mercuric chloride at 100 μg/m³ (ELKINS 1961).

Some diversity of opinion thus exists as to the maximum allowable concentration of mercury and its inorganic compounds in the atmosphere until our knowledge of the toxicity to man of low concentrations of mercury in the air has been extended, the fixation of a toxic threshold value must therefore to some extent be left to individual judgement. Among the difficulties encountered in fixing unequivocally the lowest level of toxic concentrations in the air is the fact that the mildest symptoms of mercury poisoning, which are of a psychopathological character may also occur in persons who have not been exposed to

mercury Thus, TURRIAN *et al* (1956) demonstrated a causal relationship between the average mercury concentration in the atmosphere and the incidence of certain psychopathological and neurological symptoms of poisoning present in 15 out of 58 workers who had been exposed to mercury concentrations of 10–600 μg/m³ The frequency of symptoms was also abnormally high among workers who had been exposed to concentrations below 100 μg/m³ but none of the affected workers exhibited the fully developed picture of chronic mercury poisoning.

In animal experiments (rats), FRIBERG (1959) and ROTHSTEIN & HAYES (1960) observed that inorganic mercury administered parenterally accumulated in considerable quantities in the brain and was bound there more firmly than in any other organ of the body In experiments on mice performed by BERLIN & ULLBERG (1963) a single intravenous dose of $^{203}HgCl_2$ resulted in considerable accumulation, especially in the grey matter of the *cerebellum* and in the *tuber cinereum* and *corpus subfornicalis* BERLIN & JOHANSSON (1964) demonstrated that the brains of mice that had inhaled air contaminated with mercury vapour (10 mg Hg/m³ for 4 hours) contained 10 times as much mercury as the brains of mice given a similar intravenous dose of $Hg(NO_3)_2$ The rate of injection was not stated Thus, absorption of mercury from the respiratory tract seems more than absorption by other routes to lead to its accumulation in the brain. This may perhaps also offer an explanation of the correlation demonstrated between the concentration of mercury in the atmosphere and the incidence of psychopathological and neurological manifestations, including tremor

Absorption of inhaled mercury

Detailed knowledge of the extent to which mercury is absorbed from the respiratory tract will clearly be of great importance in an attempt to fix more precisely the maximum daily dose that can be tolerated during constant exposure to mercury vapour in the inspired air

It seems that the extent of absorption of mercury vapour from the respiratory tract in animals and man has not previously been clearly determined. On the basis of a comparison of the amounts of mercury excreted in the urine and the concentrations in the inspired air GÖTHLIN (1909) reported that the uptake of mercury by man was almost complete when the concentration was less than 250 μg/m³ However HOLM JENSEN (1948) contested GÖTHLIN's method of analysis and expressed the view that his determinations of the mercury concentrations of the inspired air possibly had an appreciable negative error and that absorption was in fact incomplete. In a small number of experiments performed on three volun-

teers, GERSTNER (1931) found an absorption of 34–77 % on inhalation of air containing from 10 to 100 μg/m³ In dogs breathing air containing from 3 to 26 mg mercury per m³ FRASER *et al* (1934) found a fairly constant absorption from the respiratory tract, averaging 25 % BATTIGELLI (1960) expressed the opinion that this also applies to human beings. By means of the mercury vapour detector described by WOODSON (1939), SHEPHERD *et al* (1941) performed a few experiments in which they found that the air expired by persons who breathed air with a mercury content of 60 μg/m³ did not contain mercury On the other hand, when the mercury concentration of the atmosphere was increased to 200 μg/m³ the expired air contained about 10 μg/m³ In rats exposed to an atmosphere containing 1 mg mercury per m³ GAGE (1961) found an absorption from the lungs of about 50 % HAYES & ROTHSTEIN (1962) exposed rats to an atmosphere with a mercury content of 1 4 mg/m³ for from 30 minutes to 5 hours ²⁰³Hg was added as a tracer By calculating the amounts of inhaled mercury from the minute volume of breathing (fixed in relation to the weight of the animals) and determinations of the total amounts of mercury found in the animals, the authors concluded that the absorption from the respiratory tract was complete

Material and Methods

Personal Investigations

In order to throw light on the extent to which mercury vapour is absorbed from the respiratory tract in man, I have studied in four volunteers (including myself) the ratio of the average mercury concentration in the expired air to that of the inspired air This ratio was determined at different constant concentrations of mercury in the inspired air and for roughly constant minute volumes The influence, if any of the respiratory rate on the absorption was also studied. An attempt was made to gain some impression of the variations in mercury concentration in the expired air during the individual expirations.

The mercury concentrations in the inspired air were determined both by ultraviolet photometry in a slightly modified form of the mercury-vapour detector designed by RUSSELL (cited by BROOKS & HOLMES 1958) and manufactured by Hanovia and by the chemical method previously described (NIELSEN KUDSK 1964) as a control. Only the latter method was used for determining the average concentration of mercury in the expired air

In series of previous experiments in which direct determination of the mercury concentration in expired air were performed by means of the mercury-vapour detector I had observed that the results were affected by the amount of water vapour of the expired air Further studies showed that under such conditions the results read on the detector were too low This observation probable explains why SHEPHERD *et al.* (1941) found only 5 or none at all of the inhaled mercury in the expired air they used detector of similar construction After some experimentation, I succeeded in reducing the water-vapour content of the expired air by condensation to such an extent that the negative recording of the residual water vapour by the detector became negligible and constant, without any concurrent loss of mercury in the condenser system.

The continuous direct recording of the mercury concentration in the expired air by

means of the mercury-vapour detector and connected potentiometer recorder that wa rendered possible by this method was used in all the experiments, with simultaneous collection of the expired amounts of mercury for subsequent chemical determination. However the curves obtained show only to a limited extent the fluctuations in the mercury concentration of the expired air partly because an appreciable mixture of the expired air occurs in the relatively large dead space f the condenser system, and partly because the results recorded by the detect r at flow rates below bout 2 litres per minut are little too low Likewise, it is o ly possible to determine the average concentration of mercury i the expired air with limited accuracy the basis of these curves. Nevertheless, it appears clear from such graph of th mercury concentration in the expired air directly recorded during abnormally slow nd maximal expirations (preceded by deep inspirations) that i these circumstances the last portion f the expired ai is virtually free from mercury vapou

Experimental set-up

The experiments were performed i room with net volume of 40 m³ From an adjoining room, mercury vapour was conveyed through plastic tube into the experimental room t constant rate, which was adjusted on the basis of the ventilation of the room d the concentration desired during the experiment concerned. In some so far u published experiments, I had ascertained that the volume of ai in the experimental room which is cleared of mercury every minute fairly closely corresponds t the ventilation rate f the room (in m³/min.), i.e. chemical conversion or appreciable adsorption f mercury does not occur The rate at which mercury vapour is to be conveyed int the room i order to maintain steady-state concentratio is thus equal to the product of that concentration and the ventilation rate, provided the ventilation is constant during the experimental period The air in the room was agitated by two electric fans n order to ensure an even distribution f the mercury vapour I order to bta the desired steady-stat concentration withi reasonable time, the mercury vapour was at first led into th room t a high rate When the desired concentratio was btained, the supply was adjusted to the calculated rate. When the constancy of the mercury concentration had been checked by the mercury-vapour detecto for suitable period, the tests were begun The temperature of the room was 23 ± 1

The experimental set-up employed f the determination of the mercury concentration in the expired ai is shown in semi-diagrammatic form in fig. 1 The expired ir is conducted through two condensers connected in series. Th second condenser (Friedrichs type) is immerged in a constant-temperature water bath djusted to 11 The water from the bath is re-circulated by means f a pump thro gh the Friedrichs condenser a d double-surf co condenser placed outside the water bath. Both condensers are provided with reservoir for the collection of condensed water From the condenser system the expired ir is conducted into the mercury-vapour detector which by means f a connected potentiometer recorder records the mercury concentration After the detector breathing bag of antistatic rubber is inserted into the system fo temporary collectio of the part of th expired air that is not at once passed o by the ai pump. I the same place, the ystem i provided with valve for the supply of dry mercury-free air This valve is actuated when the volum of air transported by the pump f short period exceeds that of the expired i The pump, which is operated from an a.c. stabiliser sends the air through mercury absorber previously described (NIELSEN K DAK 1964) t pressure of bout 100 mm mercury above that of the atmosphere and at a constant rate of 7 litres per minute. The flow rate is measured by a flow-meter manufactured by Fischer & Porter

As already mentioned, the Hanovia mercury-vapour detector was used in slightly

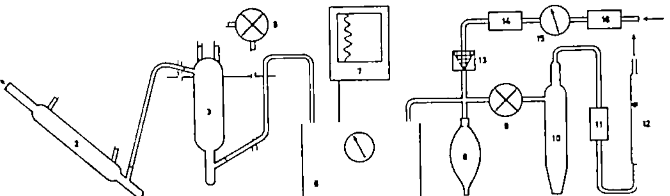


Fig. 1 Diagram showing the experimental set-up for the determination of mercury i expired ir
1 inlet for expired air 2, double-surface condenser (Quickfit, Type C 5/23); 3, Friedrichs condenser (JP Pyrex" Type 1810) 4, constant-temperature water bath at 11 5, water pump 6 mercury-vapour detector ("Hanovia Type E3472) 7 potentiometer recorder ("Varia Type G-11A) 8, breathing bag 2.5 litres (astatic rubber) 9 air pump ("Reciprotor" Type 406 G) 10, mercury bsorber 11 r vessel 1., flow-meter 13 respiratory valve 14, air-drying filter (silica gel) 15 "wet" gas meter 16, mercury-absorbing iodised charcoal filter

modified form. The modifications made consisted in (1) slight change in the electric circuit for the zero adjustment, (2) power supply through an a.c. stabiliser (3) removal of the built-in pump, and (4) careful tightening of all the air-duct systems of the instrument. By these modifications and re-calibration by means of air containing known concentrations of mercury vapour it was possible to obtain sensitivity of 3 µg and measuring accuracy of ±1.5 µg/m³ when the most sensitive measuring range was used. The "Reciprotor" air pump used was completely tight, and the passage of air through it did not give rise to any demonstrable loss of mercury nor did any such loss occur in the breathing bag.

Experimental procedure

Immediately before each experiment, the mercury concentration in the air of the room was determined by means of the detector while the air was being passed through the apparatus described, with the absorber cut out. By adjusting the a.c. supply voltage to the pump the air-flow rate was set at 7 litres per minute.

Then the entire apparatus was "washed" with mercury-free air. The absorber was then inserted, and the volunteer under test now expired air through the apparatus. The amount of mercury contained in the expired air was collected in the fluid of the absorber for a period of 5 minutes. Mercury-free air which was supplied to the system only in a few experiments, was measured by means of the gas meter shown in fig. 1. At the end of the experiment, the apparatus was again washed with mercury-free air after which the absorber was again cut out, and the mercury concentration in the room air was determined just as before the experiment. In all the experiments, the volunteers inspired the air through the nose and expired it through the mouth and a short length of plastic tubing connected with the apparatus. During expiration, the volunteers closed their nostrils with their fingers.

The mercury concentration in the expired air was also recorded graphically throughout the experimental period by means of the potentiometer recorder connected with the detector (fig. 1). On the basis of the fluctuations in the mercury concentration shown by the graph, the number of expirations during the experimental period could be counted. As already mentioned, the curves obtained did not permit an accurate determination of the average concentration of mercury in the expired air and this concentration was therefore calculated on the basis of the amount of mercury collected during the experimental period and the volume of expired air measured at the ambient temperature (22 ± 1 C).

The mercury concentration in the air of the experimental room was determined at frequent intervals, both before and after the experiments, by the chemical method previously described (NIELSEN KUDSK 1964); the results of these determinations were in close agreement with those obtained by the detector. The condensed water collected from the condenser system during the experiments did not contain demonstrable amounts of mercury (dithizone determinations).

Results

The results of the experiments are shown in table 1. The volumes of expired air were determined with a margin of error of less than ±5%. The average tidal volumes (ATPS) involve errors that do not exceed ±10%. The error of the calculated amounts of expired mercury given in the table as percentages of the inspired amounts, is less than ±20% at the lowest concentrations in the expired air (about 10 µg/m³) and less than ±15% at higher concentrations.

Table 1

Results of the experiments showing the percentages of mercury absorbed during inhalation of air containing mercury at various concentrations in relation to the average tidal volumes (ATPS). The table also shows the expired amounts of mercury as percentages of the amounts inspired. These figures are taken as expressions of the percentage that a notional dead space for mercury absorption represents of the average tidal volume.

Volunteer sex, weight	Hg conc., inspired air in μg/m³	Hg conc., expired air in μg/m³	Expired Hg, as percentages of inspired Hg	Absorbed Hg, as percentages of inspired Hg	No. of expirations in 5 min.	Average tidal volume, in litres (ATPS)
F.N K.,	49.3	10.1	20.4	79.6	24	1.45
M, 92 kg	54.7	10.4	19.0	81.0	23	1.52
	47.5	10.7	[illegible]	77.5	22	1.59
	99.0	18.0	18.1	81.9	24	1.45
	109	15.0	13.8	86.2	19	1.84
	102	15.8	15.5	84.5	25	1.40
	193	28.8	15.0	85.0	20	1.75
	205	39.0	18.9	81.1	28	1.25
	210	37.6	17.8	82.2	6	1.35
	345	50.2	14.5	85.5	23	1.52
	330	60.9	18.4	81.6	25	1.40
	340	58.8	17.[illegible]	82.8	2[illegible]	1.59
B.P.,	52.0	8.6	16.5	83.5	25	1.40
M, 63 kg	52.0	9.7	18.5	81.5	31	1.13
	50	11.5	22.9	77.1	25	1.40
	98.0	14.3	14.5	85.5	24	1.46
	98.0	17.6	17.8	82.2	31	1.13
	104	18.0	17.3	82.7	20	1.75
	201	4.4	12.2	87.8	22	1.59
	193	30.1	15.6	84.4	[illegible]6	1.35
	207	33.3	16.0	84.0	31	1.13
	370	49.3	13.3	86.7	[illegible]	1.67
	345	53.8	15.6	84.4	2[illegible]	1.59
	340	5[illegible]4	15.5	84.5	25	1.40
G L.M.,	55.6	14.3	25.7	74.3	60	0.58
F 54 kg	52.0	16.8	32.2	67.8	58	0.60
	50	16.4	3[illegible]6	67.4	61	0.57

Volunteer sex, weight	Hg conc., inspired air, in µg/m³	Hg conc., expired air in µg/m³	Expired Hg, as percentages of inspired Hg	Absorbed Hg, as percentages of inspired Hg	No. of expirations in 5 min.	Average tidal volume, in litres (ATPS)
G.L.M., F 54 kg	96.0	22.2	23.1	76.9	58	0.60
	106	21.8	20.5	79.5	66	0.53
	102	25.8	25.3	74.7	65	0.54
	204	48.7	23.8	76.2	63	0.56
	201	49.5	24.6	75.4	61	0.57
	193	47.2	24.5	75.5	6[illegible]	0.57
	377	106.9	28.2	71.8	62	0.57
	350	83.2	[illegible]3.7	76.3	54	0.65
	348	83.5	23.9	76.1	59	0.59
O.C., M, 73 kg	52.0	14.0	26.9	73.1	58	0.60
	53.8	15.2	28.2	71.8	61	0.57
	50.2	14.0	28.3	71.7	52	0.67
	98.0	26.8	27.2	72.8	57	0.61
	97.0	23.4	24.0	76.0	56	0.62
	108	26.8	[illegible]4.8	75.2	53	0.66
	198	42.2	1.3	78.7	53	0.66
	198	49.6	25.0	75.0	54	0.65
	196	48.7	24.7	75.3	54	0.65
	371	79.5	21.5	78.5	54	0.64
	350	80.9	23.1	76.9	52	0.67
	340	89.4	26.2	73.8	50	0.70

A study of the results obtained shows that the extent to which absorption occurred in the individual volunteers was subject to only relatively small variations. The ratio of the absorbed to the inspired amounts of mercury seems to be largely independent of the mercury concentration in the inspired air although absorption was a little less at the lowest concentration in the inspired air (50 µg/m³), even if allowance is made for the wider margin of error of the determinations there. Adsorptive processes in the respiratory tract thus seem to be of only minor importance in the absorption of mercury. Incidentally I performed a few experiments in which air with a varying content of mercury vapour was allowed to pass in through the nostrils of a volunteer and out through the oral cavity. During this passage, the loss of mercury was about 2 / at a concentration

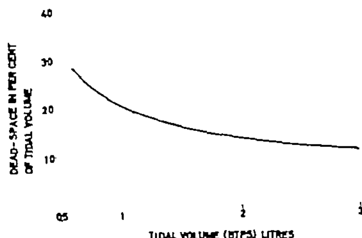


Fig. 2. Graph showing the relationship between the physiological dead space, as a percentage of the tidal volume, and the tidal volume. Based on results of ASMUSSEN & NIELSEN (1956).

of 350 µg/m³ and less at lower concentrations, as determined by means of the mercury vapour detector connected with the apparatus described above

The percentage that the amount of expired mercury constitutes of the amount inspired may be regarded as an expression of the percentage that a notional dead space for mercury absorption represents of the average tidal volume (ATPS) listed in the table ASMUSSEN & NIELSEN (1956) studied the physiological dead space as a function of the tidal volume (BTPS) in four young men On the basis of their results, I have constructed the curve shown in fig. 2, indicating the relationship between the physiological dead space, as a percentage of the tidal volume, and the tidal volume The average tidal volumes (ATPS) listed in table 1 are based on measurements at 23 of the volumes of expired air saturated with water vapour at that temperature The corresponding tidal volumes (BTPS) measured and saturated with water vapour at body temperature will thus be about 8.5 / larger

A comparison of the figures in table 1 and the curve in fig. 2 shows that the notional dead space for mercury fairly closely corresponds to the physiological dead space However at the lowest concentrations in the inspired air the dead space for mercury was a little higher in several of the experiments.

The results of the experiments thus suggest that the uptake of metallic mercury from the inspired air chiefly occurs from the alveoli of the lungs and that this alveolar absorption is almost complete and independent of variations in the mercury concentration in the inspired air within the

range from 100 to 350 µg/m³ The slightly lower uptake at a mercury concentration of 50 µg/m³ suggests that adsorptive processes are of less, or probably almost insignificant, importance in the absorption of mercury from the respiratory tract in man. A person breathing air contaminated with mercury will thus presumably take up per minute an amount of mercury that at a close approximation is equal to the product of the mercury concentration in the inspired air and his alveolar ventilation.

Discussion

Absorption is most commonly defined as the sum of all the processes by which a substance from the site of absorption enters the circulating blood. In the experiments reported here, this definition was applied to the uptake of mercury from the inspired air In experiments on rats, HAYES & ROTHSTEIN (1962) found that, after inspiration of mercury vapour (with added ^{203}Hg), temporary retention of the mercury occurs in the lungs for a few hours, after which it shows a distribution in the body similar to that observed after intravenous administration of mercuric chloride. However these authors did not determine the mercury content of the brain according to the comparative studies by BERLIN & JOHANSSON (1964) mentioned above, however the mouse brain takes up after the animal has inhaled mercury vapour an amount of mercury about 10 times as large as that taken up after intravenous administration of a corresponding dose of mercuric nitrate. CLARKSON *et al* (1961) found that metallic mercury vapour was rapidly taken up and oxidised to mercuric ions in human whole blood at physiological oxygen tensions. The oxidation mainly occurred in the erythrocytes and was rapidly followed by an equilibration of the mercury between plasma and cells. HUGHES (1957) called attention to the high solubility of metallic mercury in lipoids, compared with its solubility in water and air He expressed the view that metallic mercury – as distinct from mercuric ions, which are bound to SH-groups – readily and rapidly diffuses through lipoid-containing cell membranes (the alveolar walls), temporarily exists in its metallic form in the organism and is then dissolved in the blood lipoids and carried to sensitive tissues, such as that of the brain here the mercury is oxidised and linked up with the SH-groups of the proteins. In support of this hypothesis, HUGHES referred to investigations by BRIGATTI (1949 a & b), who both in autopsied patients and in animal experiments had found that inhalation of mercury vapour resulted in the accumulation of considerable amounts of mercury in the brain.

The processes by which mercury vapour from the alveolar air passes through the alveolar and capillary walls and the interstitial tissue into the

blood and is transported in this medium are thus not fully clarified. It may be assumed that a considerable part of the mercury vapour passes through these structures by diffusion, without being converted, and that the remainder is oxidised and temporarily deposited in these structures. Similarly it may be thought that mercury may be transported in the blood both in the form of unconverted metal dissolved in the lipoids and in an oxidised form (Hg^{++}) bound to the SH-groups of the haemoglobin and plasma albumin.

The volunteers used in the experiments reported here had been previously exposed to mercury in the inspired air only occasionally or not at all. One of the males (O C) had previously undergone unilateral thoracoplasty. All the volunteers were moderate smokers, and no restrictions as to smoking or food intake were imposed during the period of the experiments.

It should be of interest to study the absorption in persons who have been exposed to mercury vapour in the inspired air for prolonged periods. It also seems reasonable to study the factors that may be assumed to influence the absorption. Thus, I found, entirely by accident, that ingestion of alcohol to an appreciable extent reduced the absorption of mercury. This phenomenon which was studied by a slightly modified technique will be described in a paper to be submitted for publication.

Summary

Studies on the absorption of mercury vapour from the respiratory tract in four volunteers who breathed air with mercury concentrations ranging from 50 to 350 μg/m³ are reported. Determinations of the ratio of the expired to the inspired amounts of mercury gave an expression of the fraction that a notional dead space for mercury absorption represented of the average tidal volume measured during the experimental period. A comparison with the results reported by other authors on the size of the physiological dead space in relation to the tidal volume showed that the notional dead space for mercury absorption was of the same magnitude as the physiological dead space and, within the range investigated, largely independent of the mercury concentration in the inspired air. It was also demonstrated that the last part of the air expired during maximal expiration contained only negligible amounts of mercury. It is therefore reasonable to assume that almost complete absorption of mercury vapour occurs from the alveolar parts of the lungs in normal individuals.

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The Influence of Ethyl Alcohol on the Absorption of Mercury Vapour from the Lungs in Man

By

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In a previous paper I have reported the results of a study on the absorption of metallic mercury from the respiratory tract in four volunteers who inhaled air containing mercury vapour at concentrations ranging from 50 to 350 µg/m³ (NIELSEN KUDSK 1965)

In a determination of the ratio of the expired to the inspired amount of mercury I found that a notional dead space for the absorption of mercury was of the same order of magnitude as the physiological dead space and, within the range studied, largely independent of the mercury concentration in the inspired air The notional dead space for the mercury absorption was found to be dependent on the size of the tidal volume in the same way as the physiological dead space. However at the lowest concentration (50 µg/m³), the notional dead space for mercury was a little higher which indicates that mercury absorption is slightly lower than at the higher concentrations in the inspired air Adsorptive processes thus seem to be of little importance for mercury absorption from the respiratory tract. It was also demonstrated that the last part of the expired air during maximal expirations was virtually free from mercury

On the basis of these results it seemed reasonable to assume that almost complete absorption of mercury vapour occurs from the alveoli of the lungs in normal individuals. A person who breathes air contaminated with mercury vapour will thus presumably take up per minute an amount of mercury that at a close approximation is equal to the product of the mercury concentration in the inspired air and his alveolar ventilation. The processes that may be thought of importance in the absorption of the mercury vapour and its transport in the blood stream and the problems related to the fixation of a maximum allowable concentration (MAC) for mercury in the atmospheric air were briefly discussed.

The volunteers used in the experiments had previously only occasionally or never been exposed to mercury in the inspired air The experiments took place at randomly selected times within normal working hours. No restrictions on food intake and smoking had been imposed but these factors and other variable conditions of daily habits did not seem to have any effect on the absorption of mercury from the lungs However quite accidentally the author observed that the ingestion of a small amount of ethyl alcohol reduced the absorption in one of the volunteers.

The only female volunteer had ingested about 180 ml of strong beer containing about 9 g of ethyl alcohol in the next experiment, performed about 1 hour later the mercury absorption from the inspired air was 57% whereas it had previously been about 75% The experiment was later repeated with the same result. On the basis of this observation the effect of alcohol on the absorption of mercury was subjected to a closer study For practical reasons, a slightly modified experimental technique was employed

Method

Experimental set-up

As in the previous experiments (NIELSEN KUDSK 1965) I studied the absorption of mercury from the respiratory tract in volunteers by determining the ratio of the mercury concentration in the expired to that in the inspired air at constant concentrations in the inspired air and at almost constant minute volumes. The mercury concentration in the inspired air was determined by ultraviolet photometry with the same slightly modified Hanovia mercury vapour detector as in the previous experiments. This instrument in connexion with the previously described condenser system and a slightly modified experimental set-up, was also used for determining the mercury concentration in the expired air

After having passed through the two condensers, the expired air was conducted into a mixing system arranged as shown in fig. 1 From this system, the mixed expired air was passed, at a constant rate, through the mercury-vapour detector by means of a Reciprotor air pump *The rate of the air flow was measured by means of a flow-meter* which was connected through an air vessel to the outlet of the pump. The average mercury concentration in the expired air was recorded graphically by a potentiometer recorder connected with the detector Chemical control determinations of the mercury concentration in the expired air were performed at frequent intervals, as previously described (NIELSEN KUDSK 1965) In these, a mercury absorber for the collection of the mercury contained in a certain volume of expired air was inserted just after the pump The expirations were recorded by the volunteers by pressing a push-button switch through which an event marker on the potentiometer recorder was actuated.

The valve that in the previous experiment allowed inflow of mercury-free air during the experimental period was left out here. Instead, at the beginning of each experiment, the volunteer was requested to inflate the breathing bag to about half capacity by a vigorous expiration. This was done in order to avoid a disproportion between the volume of air transported by the pump and that of the expired air which might cause a negative pressure in the system and hence give rise to an error in the determination by the detector

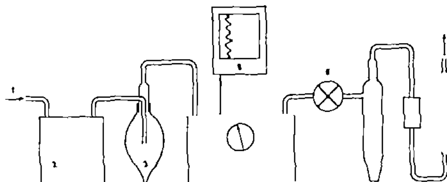


Fig. 1 Diagram of the experimental set-up used fo determining the concentration of mercury vapour in the expired air

1 inlet f expired ir 2, condenser system as before (see NIELSEN KUDSK 1965) 3 mixing system ith breathing bag (2.5 litres, astatic rubber) 4 mercury-vapour detector ("Hanovia Type E 3472) 5 potentiometer recorder ("Varian Type G-11A) 6, air-pump ("Reciprotor" Type 406 G) 7 mercury absorber 8, ai vessel 9 flow-meter

As before, the experiments were carried ut in room with net volume f 40 m³ into which mercury vapour was conducted at constant rate in order t btain a steady-state concentration. The temperature of the room was 23 ± 1 The volunteers inspired through the nose and expired through the mouth and short length of plastic tubing, which was connected with the condenser system.

By using this experimental set-up it was possible to obtain continuous, direct graphical recording of the average mercury concentration in the expired air Th concentrations determined in this way were in close agreement with the results btained by the chemical control method.

Experimental procedure

Before and after each experiment, the mercury concentration in the air of the room was determined and recorded graphically by means of the detector and the connected potentiometer recorder while the air was being passed through the entire apparatus. The air flow was set at 7 litres per minute by adjusting the a.c. supply voltage to the pump, after which the entire pparatus was washed" with mercury-free air The subject under test was requested to expire as regularly as possible through the apparatus for 3½ minutes, the last 2 minutes of which were taken as the actual experimental period. The verage mercury concentration in the expired ir was recorded as a function of time during the test by means of the recorder The expirations were also marked on the graph paper as already described.

During the chemical control determinations, th bsorber was inserted after the pump and the experimental period extended to 4–5 minutes. At the end of the experiment, the entire apparatus was again washed with mercury-free air

On the basis of these determinations the ratio f the mercury concentration in the expired air to that of the inspired ai was calculated as percentage. By subtracting this percentage from 100, the amount of bsorbed mercury was fou d as percentage f the inhaled amount. Th verage tidal volume was calculated by dividing the volume of i expired during the experimental period (measured t 23 ± 1°) by the number of expirations.

The volunteers were the same persons as those used in the previous experiments. All the experiments were performed t constant concentration of mercury in the inspired air viz.

Table 1

Results of three experiments showing the relation between the average tidal olume and the amount of expired mercury as percentage of the inspired mount, and the mercury absorption calculated on this basis in a male volunteer (F.N k.) who had not taken alcohol. The mercury concentration in the expired air was determined both by the physical method (Ph) described in this paper and by the chemical method (Ch) previously described (NIELSEN KUDSK 1964).

Hg conc., insp. air µg/m³	Hg conc., exp. air µg/m³	Exp Hg, per cent of insp. Hg	Hg absorbed, per cent	No. of expirations in 5 minutes	Average tidal volume, litres (ATPS)
187 (Ph)	28 (Ph) 30.6 (Ch)	15.0 (Ph) 16.4 (Ch)	85 0 (Ph) 83 6 (Ch)	22	1 67
211 (Ph)	38 (Ph) 39.8 (Ch)	18.0 (Ph) 18.7 (Ch)	82.0 (Ph) 81 7 (Ch)	36	1 01
204 (Ph)	57 (Ph) 54... (Ch)	?7.9 (Ph) 26 5 (Ch)	72.1 (Ph) 73.5 (Ch)	84	0 44

bout ?00 µg/m³ The absorption of mercury vapour was determined in the individual volunteer immediately before the ingestion of dose of ethyl alcohol, and subsequently at suitable time intervals, iz. every 15 minutes during the first part of the experiment and later t 30-minute intervals. The dose of alcohol was I gested over a period of about 10 minutes. In some of the experiments, the volunteers had not had any food immediately before and did not take any during the experiment. In other experiments, the volunteers had the r ordinary meals, coffee o tea during the experimental period. The doses of lcohol were given either as pure alcohol diluted with sucrose-containing soda water o in the form of strong beer

In some dditional experiments, volunteer breathed air that had first been bubbled through 25 % ethyl lcohol solution (/)

The mercury vapou detector is only slightly sensitiv to alcohol vapour the concentrations of alcohol occurring in the expired air were so low that they were not recorded by the instrument.

I these experiments the uthor did not have the opportunity to determine the alcohol concentrations in the blood of the volunteers or in the expired air

Results

Table 1 shows the results of an experiment in which the volunteer had not ingested any alcohol The figures show close agreement with the determinations of the mercury concentrations performed on expired air by means of the physical method described above and the chemical method used as a control The results also show a distinct correlation between the average tidal volume (ATPS) and the expired amount of



Fig. 2. Curve obtained by means of the potentiometer recorder showing the influence of the tidal volume on the mercury concentration in the expired air. The minute volume was 7 litres and the mercury concentration in the inspired air 204 μg/m³

mercury as a percentage of the inspired amount and hence the absorption in the individual volunteer. The expired amount of mercury calculated as a percentage of the inspired amount may be taken as an expression of the percentage that a notional dead space for mercury absorption represents of the average tidal volume.

Fig. 2 shows a curve reproduced from a direct graphical recording of the average concentration of mercury in the expired air made by the method described. The minute volume was 7 litres during the determination, and the influence of the tidal volume is distinctly apparent from the curve.

Curve I in fig. 3 shows the changes in the mercury absorption related to time in a male volunteer (F.N.K., weight 92 kg), who at zero time ingested 20 g ethyl alcohol diluted with sucrose-containing soda water. One hour previously the volunteer had had a light breakfast, but he did not take any food during the experiment. The average tidal volumes measured in the individual experiments varied between 1.1 and 1.4 litres. The curve is not corrected for the effects of these variations on the mercury absorption, which is negligible as compared with that of the alcohol. Curve II shows the results of a similar experiment in the same volunteer who had then ingested 24 g alcohol diluted with soda water. The average tidal volumes varied between 1.0 and 1.5 litres. The results shown in curve III were obtained in another male volunteer (B.P., weight 63 kg) to whom a dose of 24 g alcohol had been administered as described above. It was later disclosed that he had had a fairly heavy breakfast 1 hour before, probably because he knew that the experiment would be of relatively long duration. The variations in his tidal volumes ranged from 1.2 to 1.5 litres.

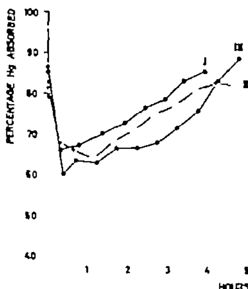


Fig. 3 Curves showing changes with time in mercury absorption in volunteers who at zero time ingested a dose of alcohol and then fasted during the experimental period.

Curve I F N K M 92 kg, 20 g alcohol.
Curve II F N K M 92 kg, 24 g alcohol.
Curve III B.P M, 63 kg, 24 g alcohol.

The experiments clearly show that the ingestion of alcohol exerts an appreciable effect on the mercury absorption from the respiratory tract in man The doses administered would presumably have caused maximum blood concentrations of alcohol of about 0 02 / and 0.025 / in the first volunteer (F N K Curves I and II) and of about 0 04 / in the other (B P., Curve III), provided that the entire amount of alcohol had been absorbed. The curves I–III thus seem to show that there is a correlation between the concentration of ethyl alcohol in the body water and the inhibition of the absorption of mercury vapour This phenomenon will later be subjected to a closer study

The three curves in fig. 4 show the results obtained in three volunteers who ingested 560 ml of strong beer i e a dose of about 27 g alcohol The volunteers had their normal meals, coffee and tea before and during the experiments Thus, they had all had breakfast about an hour before and had lunch 60–75 minutes after the experiments were begun Curve A shows the results of an experiment with the same volunteer (B.P) as had been used in the experiment illustrated in curve III The average tidal volumes varied between 1 25 and 1.55 litres. The experiment shown in curve B was performed on a male volunteer (O C) weighing 73 kg. The



Fig. 4. Curves showing changes with time in mercury absorptio[n in] volunteers who at zero time ingested 560 ml of strong beer (about 27 g [a]lcohol) and 1 hour later had normal lunch.

Curve A B.P M 63 kg.
Curve B O C., M, 73 kg.
Curve C G.L.M., F 54 kg.

range of the average tidal volumes was from 0.55 to 0 70 litre The female volunteer (G L.M., 54 kg), whose thirst for beer accidentally led to this study was subjected to the experiment illustrated in Curve C. The average tidal volumes varied between 0 55 and 0.65 litre during the experiment It appears from the curves that maximum inhibition of the mercury absorption occurred at a time at which it must be assumed that the blood con centration of alcohol was also at its peak. The greatest inhibition was seen in the female volunteer who presumably had the highest blood concentra tion of alcohol. It is a striking feature in two of the experiments (curves A and C) – veiled to some extent in the third (curve B) – that the intake of lunch caused a further secondary inhibition of the mercury absorption This observation was confirmed in several subsequent experiments. The experiments thus show that the ingestion of a moderate dose of alcohol in connexion with or between normal meals causes a prolonged and appreciable reduction in the absorption of mercury vapour from the lungs.

In similar experiments it was shown that the intake of an ordinary lunch did not affect the absorption of mercury in subjects from whom the effect of alcohol was absent.

As already mentioned, the mercury absorption was also studied in one volunteer (F N K) who breathed through the mouth mercury-containing air that had first been bubbled through a 25 / aqueous solution of ethyl alcohol (v/v). The use of alcohol solutions of higher concentrations led to discomfort in the volunteer to such an extent that further experiments had to be given up In these experiments, which were also performed at a constant mercury concentration of about 200 μg/m³ in the inspired air it was observed that the absorption from a normal initial level of about 83 / of the inspired amount of mercury fell to about 77 / within 1½ minutes, and then gradually to about 72 / within the next 10 minutes, at which time the experiments were stopped. Unfortunately it was not possible to determine the alcohol concentration in the inspired air but it was at the limit where it caused local irritation of the bronchial mucosa. These experiments suggest that the effect of alcohol on mercury absorption is not exclusively confined to the pulmonary structures If this had been so it must be presumed that the effect on the absorption would have occurred more rapidly and been more pronounced.

The error of the average tidal volumes (ATPS) listed in table 1 is less than ± 7 / The figures indicating the amounts of expired mercury as percentages of the inspired amounts involve errors that do not exceed ± 10 /

The error of the percentages showing the mercury absorption in the curves in figs. 2 and 3 is less than ± 5 / As already mentioned the curves were not corrected for the relatively slight effect of the small variations in the tidal volumes on the absorption of mercury

Discussion

The processes by which mercury vapour passes from the alveolar air through the alveolar and capillary walls and the interstitial tissue into the blood and its transport in this medium are as yet incompletely known. It may be presumed that a considerable part of the mercury crosses these pulmonary structures in the unconverted form by diffusion, and also that some of the metal is oxidised and temporarily deposited in these structures. HAYES & ROTHSTEIN (196?) found, in experiments on rats, that inhalation of mercury vapour (labelled with ^{203}Hg) gave rise to a certain pulmonary retention of the metal for a few hours, after which it was distributed in the body in the same way as after intravenous administration of mercuric chloride Approximately ?0 of the total body burden was found to be deposited in the lungs after exposure to mercury vapour for 1 hour However these authors did not study the content of mercury in the brains of the animals BERLIN & JOHANSSON (1964) found

that the mouse brain takes up 10 times as much mercury after inhalation of a dose of mercury vapour as after intravenous injection of an equivalent dose of mercuric salt HUGHES (1957) advanced the hypothesis that metallic mercury (as distinct from mercuric ions), because of its higher solubility in lipoids than in water and air readily and rapidly diffuses through cell membranes (alveolar and capillary walls) and temporarily exists in its metallic form in the body HUGHES thus held the view that metallic mercury dissolved in the blood lipoids, can be transported to sensitive tissues, such as that of the brain here the mercury is oxidised and linked up with the SH-groups of proteins However CLARKSON *et al* (1961) found that mercury vapour is rapidly taken up and oxidised to mercuric ions in human whole blood at physiological oxygen tensions. This oxidation mainly occurred in the erythrocytes, but was rapidly followed by an equilibration of the mercury between the plasma and other blood cells in it. The mercuric ions are presumably bound chiefly to the SH-groups of the haemoglobin and plasma albumin.

ANTHONISEN & CRONE (1956) found a high capillary permeability to ethyl alcohol in the lungs, shown by a high initial extraction of the alcohol from the arterial blood. As the distribution coefficient for ethyl alcohol between air and blood is about 0 0005 (LILJESTRAND & LINDE 1930) it must be assumed that the alcohol concentration in the extravascular water of the lungs is nearly equal to that of the blood Both in animal experiments and in human therapy LUISADA (1950a & b) and GOLDMANN & LUISADA (1952) showed that inhalation of ethyl alcohol vapour can prevent the occurrence of experimentally produced pulmonary oedema and has an inhibitory effect on the further development of existing oedema. The authors were unable to explain the mechanism involved, but thought that the explanation should be sought in the local anti-foaming effect of alcohol, since large oral or intravenous doses were required to produce a similar effect. In a study of the literature, I have been unable to find any specific evidence to support the assumption that alcohol exerts an appreciable effect on pulmonary vascular resistance

Like other narcotics (anaesthetics), ethyl alcohol exerts a certain surface activity and *must be presumed to affect the permeability of the cell* membranes. In the study reported here it was demonstrated that the effect of inhaled alcohol vapour on the absorption of mercury was less pronounced than that of moderate doses of alcohol taken by mouth This may suggest that alcohol has only a relatively slight effect on the permeability of the pulmonary structures to mercury vapour In view of the results reported by CLARKSON *et al* (1961), it may be thought that a reduction in the permeability of the erythrocytes produced by alcohol may be a possible cause of the diminished mercury absorption. Conside-

ring that the distribution coefficient for alcohol between lipoids and water is small (about 0.03), the possibility that alcohol inhibits the solubility of metallic mercury in (or its penetration into) the lipoids of the plasma seems less reasonable, but it cannot be rejected. HAYES & ROTHSTEIN (1962) found that mercury absorption in rats anaesthetised with pentobarbital (mebumal NFN) was less than 50 / of the amounts absorbed by unanaesthetised animals. The authors concluded that the reduction was due to a lowered respiratory rate induced by the anaesthesia, but the minute volumes in the anaesthetised animals were not measured in the experiments. It is thus uncertain if the greatly reduced absorption of mercury was in fact due to a diminished minute volume. It is possible that pentobarbital (mebumal NFN) has an effect on mercury absorption similar to that of ethyl alcohol demonstrated in the study reported here

A closer study of the mechanism of action of ethyl alcohol (and possibly other narcotics) as inhibitor of the absorption of mercury vapour thus seems desirable. The question whether ingestion of alcohol gives rise to changes in the distribution and fate of mercury in the organism should also be investigated

The toxic threshold limit value of 100 μg/m^3 for mercury vapour and inorganic mercury compounds maintained in the U.S.A. was originally fixed on the basis of extensive studies within the field of industrial hygiene performed by NEAL *et al* (1937 & 1941) A considerable number of the workers (fur-cutters and hatters) studied were of South or South-Eastern European descent, and it is scarcely unreasonable to assume that many of these workers habitually used alcohol in moderate amounts, although this was not stated On the other hand, it was reported that about 10 / of the workers suffering from chronic mercury poisoning were heavy drinkers. Excessive use of alcohol was seen in 10 / of the unaffected fur cutters, but only in about 2 / of the unaffected hatters. As contrasted with several of the general practitioners in the district concerned NEAL *et al* did not believe that alcoholism predisposed to the development of chronic mercury poisoning, but thought that the causal relationship if anything, was the reverse.

If it be assumed that a considerable number of these workers consumed alcohol habitually it may be suspected – on the basis of the results of this study – that the frequency of chronic mercury poisoning observed was lower than it would have been if they had not indulged in drinking.

In view of (1) the above considerations, coupled with the demonstration of almost complete absorption of mercury vapour from the alveolar parts of the lungs in normal individuals, (2) the observation by other authors that absorption of mercury vapour from the lungs leads to distinct accumulation of mercury in the brain of experimental animals

(BERLIN & JOHANSSON 1964) and (3) the abnormally high frequency of certain psychopathological and neurological symptoms of poisoning in workers exposed to mercury concentrations in the air lower than 100 $\mu g/m^3$ (TURRIAN *et al.* 1956), I suggest that it would be reasonable to consider a reduction of the toxic threshold limit value at present accepted for mercury vapour in the air. The toxic threshold limit value might temporarily be fixed at 50 $\mu g/m^3$ until our knowledge of the toxicity to man of low mercury concentrations in the air has been extended.

Summary

A method for direct, continuous graphical recording of the average concentration of mercury vapour in the expired air is described. By means of this method the effect of the tidal volume on the absorption of mercury vapour from the lungs is demonstrated. The results of the experiments also show that ethyl alcohol, given by mouth in moderate doses, has a distinctly inhibitory influence on the absorption of mercury from the lungs. In volunteers who had consumed alcohol – and only in such persons – food intake caused a secondary inhibition of mercury absorption. The mechanism of this effect is as yet unknown.

In view of the reduced absorption of mercury vapour demonstrated in subjects who have ingested moderate doses of alcohol and the demonstration of almost complete absorption of mercury from the alveolar parts of the lungs in normal individuals, coupled with the observation by other authors that this absorption in experimental animals leads to a distinct accumulation of mercury in the brain, it is suggested that it would be reasonable to consider a reduction of the toxic threshold limit value at present accepted for mercury vapour in the air.

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Distribution and Biliary Excretion of Decamethonium in Doubly Nephrectomized Rabbits

By

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(Received June 1 1965)

It is generally accepted that cell membranes are almost impermeable to salts of quaternary ammonium compounds, which are therefore distributed in the extracellular fluid. It has been shown in a preliminary study (BROEN CHRISTENSEN & SCHOU 1963) that the fate of intravenously injected decamethonium in doubly nephrectomized rabbits is not explainable as an equal distribution through the extracellular space After injection of ^{14}C-decamethonium a steep fall in radioactivity in plasma was demonstrated within one hour of the injection This was succeeded by a slower fall linear on a semilogarithmic co-ordinate system, within an experimental period of six hours Calculation of the volume of distribution from the plasma decamethonium level six hours after the injection gave values of about 100% of the body weight (amount of compound injected divided by the concentration in the plasma) On the assumption that decamethonium is not metabolized, this indicates an unequal distribution, with accumulation of decamethonium in one or more of the organs. The hypothesis was advanced on the basis of these results that the initial steep fall in plasma level of decamethonium might be due to a specific uptake in the motor end-plate region (cf WASER & LÜTHI 1957) and that the slow fall might be due to biliary excretion of decamethonium.

An account will be given below of a series of investigations made for the purpose of studying the factors affecting the decamethonium concentration in plasma after intravenous injection of ^{14}C-decamethonium into doubly nephrectomized rabbits. The study comprises investigations not only of the plasma decamethonium level, but also of the decamethonium concentrations in various organs and in bile at different times after the injection.

Methods

Surgical technique

In all, 16 male albino rabbits were used, ranging in weight from 1850 to 2785 g. In the first six experiments $N_2O + O_2$ + ether anaesthesia was employed. In the next halothane (fluothane ®) was used instead of ether. The operation was initiated by insertion of an endotracheal tube. Next polyethylene tubing was inserted in the left carotid artery, which was used for both blood pressure measurement and arterial sampling. Double nephrectomy was carried out via incisions i the right and the left lumber regions. The renal capsule as carefully detached, and the ureter, the renal artery a d the renal vein were isolated separately subjected to double ligation, and divided. The wounds were sutured in layers.

I fo experiments a PVC drain was inserted in the common bile duct about 1.5 cm from the opening of this into the duodenum. The bile duct was ligated at the duodenum, fter which the gall-bladder was emptied by compression. A free outflow of bile as secu ed by passing the drain declivously out into the right lumbar region. I these four animals the double nephrectomy as well as the bile duct operation were performed through n abdominal median incision. The pulse rate, blood pressure, respiratory rate nd respira t ry v lume were recorded continually from the conclusion of the operation u til 15–20 minutes after the injection of decamethonium.

Preparation injected

We used [14]C methyl-decamethonium dibromide (Radiochemical Centre, Amersham, England) with a specific activity of 12 µc/mg, received in solid form in sealed glass ampoules. Immediately after the receipt a stock solution was prepared in redistilled water, which contained 20 µc/ml a d was stored at 4°. A carrier free solution for injection was prepared f om the stock solution. This contained 1.2 µc or 100 µg decamethonium bromide/ml. The dose wa always 100 µg/kg, injected into the marginal ear vein in the course of 15–20 seconds.

Measurement of radioactivity in plasma

The arterial blood samples were collected in heparinized centrifuge tubes, about 1.2 ml per sample, and centrifuged immediately after. After 500 µl of plasma had been pipetted off the proteins were precipitated with zinc hydroxide (SOMOGYI 1930) and centrifuged down. Of the clear supernatant 500 µl were pipetted i t counting tubes containing 10 ml of scintillation medium (BRAY 1960). The additio ga rise t a light precipitate which had settled after one hour's standing. The radioactivity of each sample was measured by means of Packard Tri-C rb liquid scintillation spectrometer model 314 EX. The efficiency of the measu ements s controlled by comparison with standard samples prepared by dding know amounts of [14]C-decamethonium to rabbit plasma, which was subjected to th process described. The amount f decamethonium dibromide (molecular weight 418) in the plasma sample expressed in µg per ml plasma, w s calculated from the radioactivity measurement.

Measurement of radioactivity in tissue specimens

One hour four hours after the injection of decamethonium the animals were killed by insufflating i nto the marginal ea vein. The tissue specimens, weighing bout 1 g, were excised. Specimens of the diaphragm nd the sternomastoid muscle were prepared ith the fibres at thei full lengths. Blood on the surface of the specimen was carefully wiped off

ith filter paper. The same was done with gastric and intestinal contents. The specimens were weighed immediately after their excision. Then 0.5 N NaOH was added, the volume corresponding in millilitres to three times the weight of the specimen in grams. The mixture was left in stoppered flasks until the next day when specimens were hydrolysed by heating to about 90° for 20 minutes with repeated vigorous shaking. After cooling, the proteins were precipitated by adding a 10 % solution of $ZnSO_4 \cdot 7H_2O$ in volume the same as that of the previously added NaOH. This procedure is that employed by SUND & SCHOU (1964) for muscular tissue. After centrifugation, 500 µl of the clear supernatant was pipetted into 10 ml of scintillation medium. The radioactivity of each muscle specimen was measured in the same apparatus and by the same procedure as employed for the plasma samples. The counting efficiency was controlled by comparison with standard muscle specimens, and the content of decamethonium dibromide was calculated and expressed in ng/g wet weight of the specimen. The radioactivities of the other organ specimens were measured by means of an IDL Liquid Scintillation Counter type 6012. The counting efficiency was here controlled for each specimen by renewed measurement 30 minutes after adding 25 µl of internal standard. We used toluene-^{14}C (NEC-103) with disintegration rate of 3.83×10^{4} dpm/ml from the New England Nuclear Corp. On the basis of measurements of efficiency the content of decamethonium dibromide in the specimens was calculated and expressed in ng/g wet weight of the specimen.

Measurements of radioactivity in bile

The radioactivity of the bile samples, which were collected in fractions, were measured in a gasflow-counter with thin window model Risø. One ml of bile from each fraction was evaporated in an aluminium dish and measured for 20 minutes. The radioactivity of the sample was calculated by comparison with counting rates for standard bile samples and converted into ng decamethonium dibromide/ml of bile.

Measurement of radioactivity in expired air

In three experiments fractional collection was performed of the carbon dioxide of the expired air for periods of 20–30 minutes. The carbon dioxide was bound as ethanolamin carbonate in ethylene glycol monomethyl ether. The samples were examined for ^{14}C activity by a liquid scintillation technique based on the method described by JEFFAY & ALVAREZ (1961).

Results

Efficiency and uncertainty of the measurements of radioactivity

As the efficiency of the measurements may vary from day to day mainly owing to minor variations in the adjustment of the high-voltage supply to the photomultipliers, standard samples were prepared for each experiment. As a reference for measurement of the activity in the *plasma samples* was used the mean value of the counts for two standard samples, both containing 500 ng decamethonium dibromide/ml of plasma. For the total material the counts of the individual standard samples differed on an average by 3.2 % from the mean value, with a standard deviation of

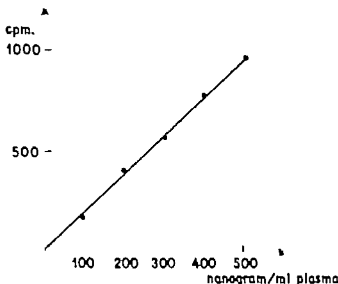


Fig. 1 Standard curv for ^{14}C-decamethonium in plasma.
Each point is the mean f r two samples.
Abscissa Counts/min te.
Ordinat Concentration of ^{14}C-decamethonium in plasma (nanogram/ml).

±1 7 / All the plasma sample counting rates were at least five times those for the plasma blanks. The standard curve being linear within the experimental range (fig. 1), there is reason to suppose that the decamethonium concentrations had no influence on the counting efficiency

By preparing the striated *muscle specimens* as described above, we obtained a constant composition for these even though they had differed in weight when excised, from 0.8 to 1 1 grams References and blanks were prepared on the analogy of those for the plasma samples Not less than 5000 counts have been recorded per specimen As however the counts were no more than 3 or 4 times the background count, the uncertainty was somewhat greater than that found for the plasma samples. The muscle standard curve, which has not been reproduced here is linear within the experimental range.

Measurement of the *organ specimens* containing the least radioactivity gave counting rates that were only about twice the background count, so that the uncertainty in relation to the background count became of increased importance In such circumstances the statistical error for 5000 counts recorded per specimen will be 4–5 / To this must be added the error of the efficiency determination after adding the internal standard, which amounts to 1–2 / Use of an internal standard for calculating the

counting efficiency of the specimen presupposes that the latter is not altered by addition of the internal standard (SCHRAM 1963) It has been shown, in unpublished experiments, that the counting efficiency remains unchanged until more than 30 µl of toluene is added to 10 ml of scintillation medium. As background we used, for practical reasons, the number of counts observed for 10 ml of scintillation medium to which had been added 500 µl of water instead of a blank, the latter corresponding in colour and chemical composition to the organ specimen (DOMER & HAYES 1960). This error is, however of little importance, because the efficiency varies a few per cent only from one organ to another and is of the same order as that measured after adding internal standard to 500 µl of water in 10 ml of scintillation medium (40–45 %)

The justification for comparing the results of the plasma tests with those calculated by means of internal standard was assured by measuring the efficiency on counting the standard plasma samples after adding internal standard. Calculated on this basis, 91 to 97 % of the added activity was recovered

The radioactivity of the bile samples could not be measured by liquid scintillation owing to pronounced colour quenching. The measurements were carried out at the Danish Atomic Energy Research Establishment, Risø, in an apparatus for measuring samples with a small content of β-emitters. The background counting rate was low (0 8 counts per 20 minutes) and the counting rates for the samples were not less than 20 times the background counting rate

Plasma level of ^{14}C-decamethonium

The plasma decamethonium levels in two groups were compared, as shown in table 1 One group had been anaesthetized with ether and the

Table 1

Concentration of decamethonium in plasma 10 and 60 minutes after intravenous injection of 100 µg/kg into two groups of rabbits, one anaesthetized with ether the other with halothane.

The results are given as mean values ±SD

Anaesthetic	Number of experiments	Concentration (nanogram/ml) 10 min. after injection [1]	60 min. after injection
Ether	6	632 ± 70	341 ± 40
Halothane	6	511 ± 61	302 ± 29

[1] Difference between the two groups is significant (P < 0.01)

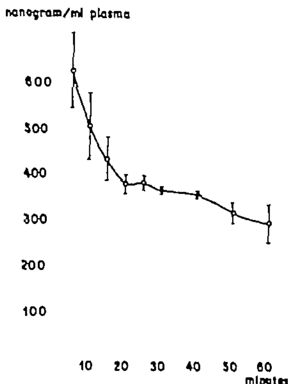


Fig. 2. Concentration of decamethonium in plasma after intravenous injection of 100 μg/kg.
Each point is the mean of values from 4–5 experiments.
The vertical bars indicate the standard deviation.
Abscissa Time in minutes after injection.
Ordinate Concentration of decamethonium in plasma (nanogram/ml)

other with halothane In both groups administration of the anaesthetic had been discontinued 5 minutes before the injection of decamethonium. The biological effect, manifesting itself as a minor reduction in the respiratory volume of a few minutes duration was the same in the two groups. The plasma concentration of decamethonium, on the other hand, measured 10 minutes after the injection, was significantly higher in the ether than in the halothane group There was only a slight difference between the two groups 60 minutes after the injection.

The results of decamethonium determinations on plasma were in agreement with those previously published Fig. 2 is a graphic representation of the results of five experiments, in which the plasma decamethonium levels within one hour of injection were determined. The experimental animals were under halothane anaesthesia. The curve for the decamethonium concentration shows a steep fall during the first 20 minutes. For

the period from 20 to 40 minutes after the injection the curve runs an almost horizontal course, thereafter falling slowly By calculating the volume of distribution on the basis of the plasma decamethonium level during the period from 20 to 40 minutes after the injection, we arrive at a value constituting about 27 / of the body weight.

In our study the blood analyses covered only arterial blood. In a preliminary investigation the decamethonium concentration was followed for one hour after the injection in samples withdrawn simultaneously from the carotid artery and the ear vein After rapid intravenous decamethonium injection higher concentrations were found in plasma from arterial blood than from venous blood during the first 5–10 minutes, whereas after that there was no significant difference

Distribution of ^{14}C-decamethonium in tissues

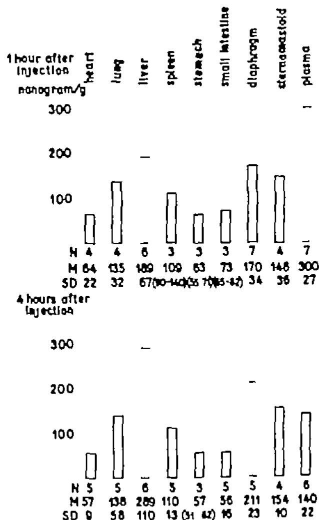
Fig. 3 illustrates the results of the organ analyses compared with the corresponding plasma decamethonium levels. One hour after the injection the striated muscles were found to contain decamethonium at concentrations more than twice as high as those in cardiac muscles and in organs consisting mainly of smooth muscles (small intestine and stomach) The liver spleen and lung tissues contained decamethonium at concentrations of the same order as did striated muscles Four hours after the injection the decamethonium concentration in the striated muscles had risen a little more, whereas the concentration in the heart, lungs, spleen intestine and stomach were the same as one hour after the injection. The liver was the only organ showing a considerably higher decamethonium concentration than that in plasma.

Biliary excretion of ^{14}C-decamethonium

Bile was collected in fractions from four of the experimental animals in the series examined for distribution in organs The results are shown in table 2, in which the decamethonium concentrations in bile have been set out along with those in plasma. It is seen that the decamethonium concentrations in bile were lower than those in plasma throughout the experimental period, though with a hardly significant difference during the period from three to four hours after the injection The average excretion of ^{14}C constituted 1 9 $\pm$ 0.5 / of the dose given

$^{14}CO_2$ in the expired air

Within the first hour after injection of decamethonium $^{14}CO_2$ was not detectable with certainty in the expired air During the next few hours, on the other hand, an increasing excretion of $^{14}CO_2$ was demonstrable Th



Figur 3

F g. 3 Concentration of decamethonium n tis ues and plasma one nd fou hours after ntravenous injection of 100 µg/kg
Vertical scale Concentration of decamethonium expressed a g.g wet weight of tissue and g ml of pla ma.
N mber of animal (N) and mea values of concentrations (M) ith corresponding standard deviation (S D) are indicated below the columns Figures in bracket indicate range

Table 2

Concentration of decamethonium in bile and plasma after intravenous injection of 100 μg/kg. Bile was collected for four one-hour periods. The values fo plasma indicate the concentration t the end f each one-hour period. Results are expressed as the mean of fou experiments ±SD

One-hour periods after injection	0–1 hours	1–2 hours	2–3 hours	3–4 hours
Concentratio in bile (g/ml)	233 ± 58	175 ± 37	150 ± 34	119 ± 31
Concentration in plasma (ng/ml) at the end of each period	361	.61	182	153

total excretion of ^{14}C within four hours constituted, however less than 0.5% of the injected amount

Discussion

The experiments showed a significantly higher decamethonium concentration in plasma after ether anaesthesia than after halothane anaesthesia within 10 minutes of the injection. Any evaluation of the effect of the anaesthetic agent itself on the distribution and action of decamethonium is, however not possible, as the anaesthetic was not maintained throughout the experiment. The abovementioned difference between the two groups may have been due to a difference in anaesthetic levels.

The results of the ^{14}C-decamethonium determinations in plasma are in fair agreement with those of GIOVENELLA, MANNI, MAZZONI & MORICCA (1961) in experiments on intact rats they found a steep fall of the plasma curve and then a small secondary peak, after which it followed an almost horizontal course. We know from WASER & LÜTHI s (1957) autoradiographic studies of mouse diaphragms that radioactivity accumulates in the region of the motor end plate a few minutes after intravenous injection of ^{14}C-decamethonium. This accumulation in connection with a distribution of decamethonium in the extracellular space explains the steep fall of the curve in fig. 2 during the first 20 minutes after the injection The secondary peak is low the 25-minute value differing from the mean at 20 minutes by less than its standard deviation.

GIOVENELLA *et al* (1961) found the secondary peak to coincide with the beginning of recovery of neuromuscular transmission, and they took the phenomenon to indicate a liberation of inactivated decamethonium from the receptor The results of our investigation can neither bear out nor

refute this hypothesis, but it is evident that the shape of the curve for the period from 20 to 40 minutes after the injection reflects at least two different transfer functions A steady state is out of the question as the curve has a clear descending tendency after 40 minutes.

One hour after the injection about 150 ng ^{14}C-decamethonium were recovered per gram striated muscle. With a muscular mass of about 50/ of the body weight, this corresponded to about 75/ of the dose injected The total uptake of decamethonium by striated muscles cannot be settled, because the decamethonium concentrations differ appreciably in the various muscle groups The extracellular space of the rabbit diaphragm does not exceed 0.3 ml/g, assessed on the basis of the Cl distribution volume (MANERY & HASTINGS 1939) The decamethonium concentrations found must therefore indicate a binding on the cell membrane or invasion of the muscle cells. During the period from one to four hours after the injection the decamethonium concentration in striated muscles rose only slightly At the same time, however a halving occurred of the concentration in plasma and thus presumably also of that in the interstitial fluid of the muscles. This suggests a continued uptake of decamethonium by the muscle cells. CREESE, TAYLOR & TILTON (1963) in *in vitro* experiments with striated muscles from guinea pigs, rabbits and rats, studied the uptake of radioactive iodocholinium, a bisquaternary ammonium compound having the same biological action as decamethonium In experiments extending over several hours iodocholinium was shown to be taken up in an amount greatly exceeding that to be expected if the agent had merely diffused into the extracellular space

We have no evidence that decamethonium can penetrate into the muscle cell *in vivo* WASER s (1962) autoradiographic technique does not allow us to distinguish between binding on the cell membrane and invasion of the cell. WASER (1962), in a contribution to a discussion on a symposium on curare and curare-like agents, London 1961 suggested that decamethonium changes into a lipophil compound by a metabolic process and that this might explain the penetration into the cells.

McISAAC (1962) studied the distribution of ^{14}C hexamethonium in doubly nephrectomized cats and found ^{14}C concentrations in plasma and tissue suggesting slow penetration of hexamethonium into the cells.

Our investigations showed that there was no question of a concentrating transfer of decamethonium from plasma to bile This is in agreement with the results reported by SCHANKER (1962). The excessive accumulation of ^{14}C demonstrable in liver tissue four hours after the ^{14}C-decamethonium injection suggests a metabolic process in this organ. The study under review did not include an investigation into the question whether the ^{14}C activity in plasma, bile and organs represents unchanged decametho-

nium. The presence of small but increasing amounts of $^{14}CO_2$ in the expired air during the first four hours after the injection suggests a slow metabolism of decamethonium. The radiochemical purity of the preparation for injection was controlled by paper chromatography and the preparation was found not to be degraded by storage for several months in aqueous solution. The results of the chromatographic investigations which it is hoped to publish in connection with those of a study on the renal excretion of decamethonium, tend to suggest the presence of a metabolite, of relatively little significance quantitatively in the latter part of our experimental period.

Conclusion

When ^{14}C-decamethonium is injected into doubly nephrectomized rabbits, it is not distributed equally in the extracellular fluid. The uptake of ^{14}C-decamethonium by striated muscle has been shown to exceed greatly the capacity of the extracellular space, which can only be explained by a binding of decamethonium on the cell membranes or by invasion of the cells. An uptake has also been seen to occur in other organs. Further analysis of these findings suggests the possibility that part of decamethonium may be metabolized.

Summary

Plasma level, tissue distribution and biliary excretion were examined after intravenous injection of ^{14}C-decamethonium into doubly nephrectomized rabbits. Measurements of the decamethonium concentration in plasma showed that decamethonium was not evenly distributed in the extracellular space. During a four hour experiment an increasing uptake of decamethonium was found in striated muscles. Evidence is presented to suggest a binding on cell membranes or an invasion into muscle fibres. Throughout the experiment the concentration of decamethonium in bile was lower than in plasma. In conclusion, it is suggested that part of the decamethonium may have been metabolized.

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The Specific and Non-Specific Cholinesterase Activity in Brain and Ileum of Guinea Pigs Killed by Intravenous Paroxan at Different Infusion Rates

By

Jens Jensen-Holm

(Received July 23, 1965)

In the past 20 years much work has aimed at finding the degree of cholinesterase inhibition necessary to alter various biological functions (*in vivo* or *in vitro*) or to cause death. The differences in experimental conditions, in the animals used and in the methods for determining cholinesterase activity make it difficult to obtain a clear picture. However separate determinations of specific and non-specific cholinesterase activity increase the understanding of the enzymatic function (for further details, see KOELLE (1963)). In general, it is assumed that about 50–75 / of the enzymatic function must be destroyed before altered biological functions can be recorded, and most of the activity has to be destroyed to involve death (NACHMANSOHN & FELD 1947)

In the isolated rabbit duodenum SHELLEY (1955) examined the effects of physostigmine and DFP (di-isopropylphosphorofluoridate) on the increase in the muscle tonus related to [AChE] and [ChE] From the results it is seen that [ChE] has no relationship to the tonus increase, whereas [AChE] has. This conclusion was not drawn in the paper mentioned. An increase of tone in the logitudinal muscle coat appears already when less than 20 / of [AChE] is inhibited, and reaches a maximum with its total inhibition. RIKER & WESCOE (1949) studied the effect of intra arterial DFP on the cholinesterase activity of the submaxillary gland and its threshold to nerve stimulation. They found that approximately 50 / of the cholinesterase activity in the gland could be inhibited without changing the threshold for stimulation. No destinction was made between [AChE] and [ChE]. Because DFP was used, a 50 / inhibition of the cholinesterase activity at the concentration of acetylcholine bromide

Table 1

[AChE] and [ChE] in brain and ileum in controls and after lethal intravenous infusion of paroxan t different rates to guinea pigs.

The periments were carried out nder urethane naesthesia (1 5 g/kg s.c. 90 min. before). The control animals were untreated. The distribution of [AChE] nd [ChE] for this group ( ) is given at 1 mM of acetylcholine iodide, expressed as mentioned in the list of symbols.

I guinea pigs killed by different rates f paroxa infusion in the jugular vein, the [AChE] and [ChE] are expressed as percentages of the corresponding control

The different rates of infusion of paroxan (μg/min., expressed per kg body weight), the LD (in μg/kg) and the time (l min.) until death (as defined the text) are given.

Guinea pig no.	Paroxan: Concentration in the infusion fluid (μg/ml)	Paroxan: Infusion rates in μg/min. expressed per kg	LD μg/kg	Time of infusion (min.)	[AChE] brain	[AChE] ileum	[ChE] brain	[ChE] ileum	
) 11					9.92	2.39	0.478	1.79	
14					8.45	1.78	0.775	1.51	
15					9.17	2.03	0.669	1.84	
3					7.58	1.90	0.614	2.22	
22					8.09	1.29	0.704	1.65	
					8.64	1.88	0.648	1.80	mean
					0.41	0.18	0.050	0.12	± s.e.m

b)	9	37.5	5.6	518	92.7	2.0	23.5	8.9	31.2
	8	37.5	5.8	901	52.4	2.2	53.6	3.2	55.5
)	6	50	7.8	993	127.7	4.3	11.3	6.3	14.7
	24	50	16.1	1494	92.7	1.6	0.7	0.0	2.2
	22	50	16.8	1137	67.7	22.0	20.2	29.3	15.0
	28	50	17.1	1001	58.6	12.2	23.5	16.5	60.1
	16	50	29.7	1471	49.5	9.3	46.2	5.7	64.8
	17	50	32.1	1273	39.6	22.0	26.7	22.3	33.0
d)	4	100	27.4	1163	42.5	3.4	14.6	4.9	18.9
	20	100	48.0	1012	36.2	7.7	38.7	11.2	61.6
	26	100	73.5	2203	30.0	18.5	16.1	1.1	25.9
e)	10	250	36.2	649	17.9	1.0	10.5	2.8	11.7
	3	250	73.9	1243	16.4	2.4	11.9	3.5	16.7
	27	250	96.4	1371	14.2	12.5	14.9	6.0	20.5
	7	250	125.1	1797	14.3	1.0	11.1	1.5	14.5
	21	250	263.0	2063	10.9	6.7	16.9	1.5	5.3
f) [1]	11	100	26.5	983	37.1	0.1	0.4	0.7	1.6
	19	100	27.5	978	35.6	5.3	14.6	6.9	17.2
	5	100	28.5	980	34.4	3.4	24.7	7.1	43.4

activities in % of controls

[1] Pretreated with paroxan no. 11 200 μg/kg daily on 6 days until 3 days before.
– 19 235 μg/kg s.c. daily on 9 days until 1 week before.
– 5: 320 μg/kg s.c. on the day before.

used, 15 mM may permit the conclusion that [ChE] was fairly completely inhibited and [AChE] only slightly reduced. Therefore the results may be brought into line with SHELLEY's (1955) results.

According to HAWKINS & GUNTHER (1946) the non-specific cholinesterase (ChE) perhaps plays no part in the hydrolysis of acetylcholine *in vivo*. On dogs they showed that a selective complete inhibition of [ChE] in blood and organs could be established without any signs of an accumulation of acetylcholine. MAZUR & BODANSKY (1946) concluded that the toxicity of DFP could not be correlated with its effect on cholinesterases.

As pointed out by NACHMANSOHN & FELD (1947), experiments on the whole animal are not suitable for determining the minimum cholinesterase activity necessary to maintain normal function because so many centres of different sensitivity are involved. Nevertheless intact guinea pigs have been used in our study in which intravenous infusion of paroxan (diethyl-*p*-nitrophenylphosphate) was given in order to find a relationship between the rate of infusion, the time of infusion, the lethal dose (LD) and the inhibition of [AChE] and [ChE] in brain and ileum at the time of death. Various biological functions were followed during the experiments.

Methods

Biological procedure

Male guinea pigs (average weight, 578 g) were given 1.5 g/kg of subcutaneous urethane. A cannula was inserted in the trachea and connected to a Fleisch pneumotachograph. The respiratory frequency, the rate of air flow and the respiratory volumes were measured (described in detail by JENSEN-HOLM 1965a). The carotid was connected to a high pressure transducer, the blood pressure was measured and the pulse rate counted. In some experiments a rubber balloon, 20 mm long and 6 mm in diameter and filled with water was inserted in the lower part of the ileum and connected to a low pressure transducer (all transducers used were Statham models). The abdominal wall was then closed. The biological functions mentioned were recorded through an amplifier (Simonsen & Weel) connected to a jet ink recorder (Elema-Schönander) and were also followed on a four-channel oscilloscope (Simonsen & Weel, Model S-4). The temperature was kept between 37.5 and 38 and measured electrically in the pectoralis muscle. A thin polyvinyl catheter was inserted in the jugular vein and connected proximally to an apparatus for continuous infusion of paroxan dissolved in saline. In table 1 the concentrations are indicated. The infusion rates are given in µg/min expressed per kg of body weight.

The moment of death was defined as *10 seconds after respiration ceased*. The infusion was stopped at this moment, the thorax was opened, and the animal was bled by opening the heart. Then a 6–8 cm long segment of ileum, 20 cm proximal to the caecum (just distal to any inserted balloon) was taken out, prepared free from the mesentery and washed, excess water was quickly removed, and the segment was weighed and frozen to −15°. The brain (without cerebellum, medulla oblongata and nerves) was weighed and frozen. The whole procedure should usually be completed within 3 minutes to prevent either further inhibition or any spontaneous reactivation (JENSEN-HOLM 1960).

Determination of [AChE] and [ChE].

The method used for the simultaneous separate titrimetric determination of the activity of the two enzymes is described by JENSEN-HOLM (1965b). Two successive concentrations of acetylcholine (1 and 10 mM) were used.

Determination of the amount of paroxan necessary to kill the animals as described was carried out by means of an attached counter which made it possible to measure the volumes infused with great accuracy

Symbols used

AChE specific cholinesterase.
[AChE] specific cholinesterase activity in μmol/min. and gram of wet tissue.
ChE non-specific cholinesterase.
[ChE] non-specific cholinesterase activity expressed as for [AChE].
AChI acetylcholine iodide.

The figures given for the activities refer to the concentration of AChI at 1 mM. If it is desired to express these activities at 10 mM, it can easily be done by multiplying the figures for [ChE] by 1 975 and for [AChE] by 0 645 (JENSEN-HOLM 1965b).

Results

The results are presented in three parts, referring to (A) the effect of the infusion rate on the size of the lethal doses (LD) and on the time necessary to kill the animals (B), the [AChE] and [ChE] corresponding to the time of death defined above (C), a survey of the biological functions followed in the last ten of the experiments carried out

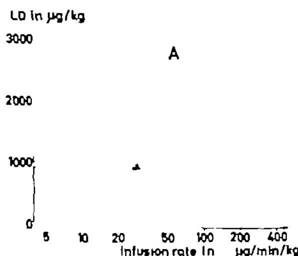
A. *The effect of the infusion rate* on the lethal dose (LD) of paroxan (in μg/kg) is shown in fig. 1 A. At increasing rates of infusion the LD rose. The values marked by crosses are from earlier experiments carried out in the same way but with a smaller dose of urethane (1.25 instead of 1.50 g/kg s.c.) It seems that urethane reduces the lethal dose of paroxan.

If the different rates are plotted against the corresponding times of infusion, a hyperbolic curve results In fig. 1 B, therefore, the rates of infusion (in μg/min expressed per kg) are plotted by their reciprocal values, but indicated by the original figures (from table 1). The time of infusion until death of the guinea pigs is plotted in minutes. The continuous oblique line is the regression line, and there is a highly significant positive correlation ($p < 0\ 001$). The equation for the curve is

$$Y = X + 7.35 \quad \text{where} \quad X = \frac{10^3}{\mu g/min. \times kg}$$

and Y = the infusion time in minutes from the beginning to the time of death.

Fig. 1 The effect of the rate of lethal paroxan infusion on the LD for guinea pigs (part A) and the infusion times (part B).



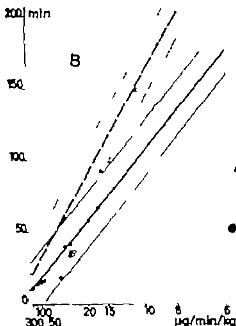
Part A Abscissa Rate of infusion in µg/min. expressed per kg body weight.
Ordinate LD of paroxan in µg/kg.
The dots represent the groups *b c*, *d* and *e* mentioned in table 1 and the open circles group *f* (pretreated with paroxan as indicated in table 1). The crosses give the results of earlier experiments, in which lower dose of urethane was used (1.25 g instead of 1.50 g/kg).

The basis for the calculation are the 16 experiments summarised in table 1 (b, c, d and e) After a preliminary calculation it was decided to exclude no 8 and 9 as being outside $\pm 1\ 96 \times s$. The regression line and the equation correspond to the 14 experiments. The greater the infusion rate, the shorter the time until death, which seems logical as later pointed out in the discussion, the greater also is the necessary LD The equation is used in table 2.

The results from the four earlier experiments, indicated in fig. 1A by crosses are also shown They appear to lie on a straight line, lying within $Y \pm 1.96 \times s$. The equation for the regression line, expressed as explained above, is

$$Y = 1\ 4 \times X + 17\ 1$$

There is a high statistical significance ($p < 0.001$) for a positive correlation.



Part B Abscissa the rate of infusion (µg/min., expressed per kg), set out on a reciprocal scale, but indicated by the original figures, taken from table 1
Ordinate time of infusion (min.) until the moment of death.
For the dots (referred as above) the continuous oblique line is the regression line, and the broken parallel lines indicate the 95 % limits of the two-dimensional distribution ($Y \pm 1.96 \times$ (standard deviation)). The two dots surrounded by a circle are excluded results (see text) For the crosses, representing earlier experiments, the same statistical procedure was used as indicated. The equations for the lines are given in the text. The open circles represent group *f* 1 table 1 (pretreated with paroxan).

The open circles correspond to the guinea pigs no 5, 11 and 19 pretreated with paroxan, as indicated in table 1 They are excluded from the calculations mentioned above. The results (LD and time of infusion) obtained in the pretreated animals were nearly identical, unlike the results from the other groups. But the figures for [AChE] and [ChE] measured nevertheless differed greatly The infusion rates used were about the same.

B. *[AChE] and [ChE].* The results of the determination of cholinesterase activity are given in table 1 [AChE] and [ChE] correspond to the activities found at 1 mM of AChI In the control group (a) the mean for 5 guinea pigs and its standard error are shown Both kinds of enzymes are present in the two organs The proportion of [ChE] is greatest in the ileum

For the four groups (b, c, d & e) of the guinea pigs killed at different rates of paroxan infusion, the activities are expressed as percentages of the corresponding control values. The activities were found to fluctuate

Table 2

Calculation of the infused amount of paroxan and the infusion time at different rates of infusion.

By use of the equation to the regression line (Y = X + 7.35 fig. 1), it is possible to calculate the lethal dose and the infusion time until death occurs. The anaesthesia was thought produced by 1.5 g/kg of urethane. In the table are given some examples of figures for different infusion rates. The central calculated values are i agreement with the experimental results obtained.

Paroxa Infusion rate, µg/min expressed per kg (arbitrary)	LD in µg/kg (calculated)	Time of infusion in min. (calculated)
3	1,022	341
10	1,074	107
30	1,221	40.7
100	1 735	17 4
300	3,205	10.7
1,000	8,350	8.4
3,000	23,050	7 7
10,000	74,500	7 6

greatly In general the inhibition was most pronounced in the brain, where on average the inhibitions of [AChE] and [ChE] were equal. [AChE] varied between 1 and 22 / of the control activity and [ChE] varied from zero to a maximum of 30 / In the ileum the inhibition was less pronounced on an average the activities were found to be 21 / ([AChE]) and 28 / ([ChE]) of the control values

In the last group *pretreated* with paroxan (group f) the activities were found to be smaller than in the other groups, especially in guinea pig no 11 in which almost no activity could be measured.

C *Biological functions* In the ten animals (no 16–28) the inspired and expired air flow (peak values) the expiratory volumes and the respiratory frequency were followed. The results are given as average values (originally and at 5 10, 20, 30 etc of the time from the beginning until death). The details and comments on them are being published separately (JENSEN-HOLM 1965a) It was shown that *the expiratory air rate* increased immediately after beginning the infusion and reached its maximum after about 60 of the time. It then decreased to less than 40 / of the initial value. *The inspiratory air rate* was slightly reduced during the first half of the experiments. Then an increase took place, and a maximum was found after 70 / of the time It was then reduced to just below the initial value. The *frequency* and the *respiratory volume per minute* were decreased

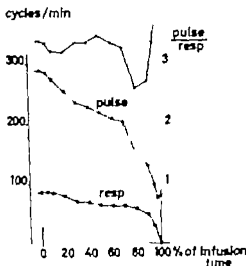


Fig. 2. The pulse frequency compared to th respiratory frequency in guinea pigs killed by intravenous infusion of paroxan.

In 5 of the guinea pigs (no. 20 to 26) it was possible t count the pulse rate throughout the experimental periods. The verage results are presented as cycles per minute, as is the respiratory frequency The upper curve shows the quotient between the frequencies of pulse and respiration. The final increase may possibly refer to the utomated heart function at the beginning of the total block seen the E.C.G

throughout the time of the experiment. *The volume per cycle* was increased somewhat during the first 70 / of the time and then decreased sharply

The blood pressure was followed in most of the experiments and found to be more or less unchanged during the experiments. *The pulse amplitudes* were reduced during the experiments, but increased greatly just before death. It should be mentioned that the heart beats continued after the respiration stopped. *The pulse frequency* determined by counting the amplitudes per 10 or 15 seconds was found to decrease during the whole period (fig. 2) to about ¼ of the initial average value. A slight increase was found before death.

The intraluminal pressure in the ileum was followed in 7 of the animals (from no 16 to 24). After about 25 / of the time, the pressure and fre quency of the peristaltic movements usually increased. During the last 20 / of the mean period no pressures could be measured.

E.C.G -changes In three experiments, carried out in 1958[1]), the electrocardiographic changes were followed during paroxan infusion until

[1]) These unpublished results re from experiments carried out with Professor Knud O. Møller and dr K. Millbers. They are not included in the general material, because the conditions used differed too much. I am grateful for permission to publish some of results.

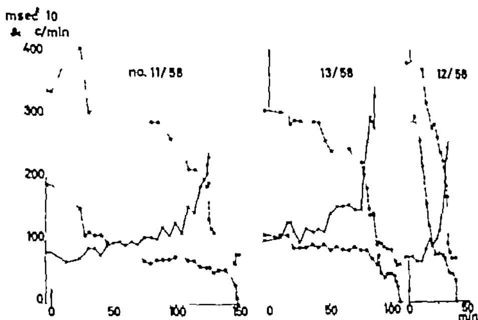


Fig. 3 E.C.G nd frequencies of heart beats and respiration fter intravenous infusion of paroxan into guinea pigs.

The abscissa Time of infusion until death in min.
The ordinate (P R) × (P–P) in (msec.)² (the fully drawn curve).
The frequencies of heart beats (—●—) and of respiration (O - -) in cycles per minute.

Results for the three experiments

P roxan in

G inea pig. no	Rat of infusion µg/min. xpressed pr kg	LD µg/kg	T me of infusion min.
11/58	8.24	1600	150
13/58	17 8	1961	108
12/58	45.6	1870	41

death. The respiratory frequency was found by counting every 4–5 minutes. The interval from P to R and from P to P were measured in msec. Both were found to increase during the experimental period In fig. 3 the slope of the product[2]) of P–R and P–P (continuous line) and the frequencies of the heart beats and the respiration are shown The product mentioned is maximally increased at the most critical part of the poisoning (at the occurrence of heart block). The frequencies of the beats and the respiration descreased throughout the period

Discussion

In this investigation whole animals (guinea pigs) were used, although, as pointed out by NACHMANSOHN & FELD (1947) such experiments are not suitable for determining the minimum activity of cholinesterases that are necessary to maintain normal functions. But if the problem is to find a possible correlation between an existing altered condition *in vivo* and a certain decrease in cholinesterase activity found, it is necessary to carry out experiments on intact animals as well. If poisoning with an anti cholinesterase agent causes death, the results of work on isolated organs will give no information adequately relevant to the problem. The question of any effect of the rate at which the inhibition takes place has first of all to be answered. Therefore, in our work the inhibitor (paroxan) was given as an intravenous infusion, and only the rate of infusion was varied. The other experimental conditions, such as the anaesthetic, the temperature and the technique, were kept more or less constant. The duration of the anaesthesia would effect the results. Therefore the rate of infusion of paroxan was kept sufficiently high to kill the animals within about two hours, in from about 11 to 128 minutes. The moment of death was defined as *cessation of respiratory function for 10 sec.* After the respiratory arrest, heart function may continue for several minutes (cf HEUBNER 1905). This information is based on experiments not included among these covered here, in which the heart was opened immediately after 10 seconds of respiratory arrest mentioned.

The specific and non-specific cholinesterase activities in the brain and ileum were determined simultaneously by a titrimetric method (JENSEN-HOLM 1965b) In the paper just mentioned, it is suggested that the ChE has no significant acetylcholine hydrolyzing action *in vivo* and thus the [AChE] must play the most important role. It is therefore necessary to separate the figures for the activities of the two cholinesterases. This can only reliably be done, if their activities are expressed by the abilities to

[2]) This product had been found empirically to give better impression of the cha ges than P–R or P–P alone.

split the physiological substrate acetylcholine. Unfortunately by this procedure it is impossible to distinguish between the part of [AChE] which is active *in vivo* and the inactive parts. Generally it is stated that only a minor fraction of [AChE] functions *in vivo* (SCHWEITZER *et al* 1939 KOELLE & STEINER 1956 KOELLE 1957) Further it is unknown how much of the active part is of true importance for biological functions.

The effects of the different rates of paroxan infusion on the doses and time of infusion is shown in fig. 1 The equation for the regression line shown, for the effect of the infusion rate on the LD and the time necessary to kill the animals, is used in table 2, where the results of some calculations are given If the equation within a defined range is approximately correct, it may be concluded that the LD increases with increasing rate of infusion. Further it is shown that there is apparently a minimum time of about 7 minutes for killing the guinea pigs. Nevertheless we know that it is possible to cause death even sooner Then factors not related to the inhibition of the cholinesterases (and accumulation of acetylcholine as a direct consequence) may play a part At exceptionally slow rates of infusion, the spontaneous reactivation of and the ageing of the enzymes (SHELLENBERGER *et al* 1965) will affect the results. Because of the limited experimental period and of the inhibitor used, these factors may play only a minor part.

In group *e* (table 1) the highest concentration of paroxan was used in the infusion fluid At an increasing rate of infusion the LD was increased and the time to death reduced If the rate of infusion (as the abscissa) and the corresponding LD (as the ordinate) are plotted on log scales the points are situated on a straight line.

In the small group *f* pretreated with various doses of paroxan on the days before, the rates of infusion used were 26.5–27.5 and 28 5 μg/min expressed per kg. The corresponding experimental periods were decreased so that the LD values were the same It would have been of interest to carry out experiments by use of more varied rates of infusion into such pretreated animals to get further information Though the activities measured generally were found to be smaller than for the non-pretreated guinea pigs, they varied rather widely The figures for the ileum in animals no 5 and 19 were rather high.

In this c nnectio it hould be noted that in one single experiment (gu nea pig no 1– not included table 1) pretreated precisely no. 11 and killed by bleed g without any prior infusion of p xan, the figures for [AChE] nd [ChE] in the ileum (identical depl ted) were found t be 1 6 nd 3 6 times the corresponding control alues This phenomeno has not been further estigated, nd we cannot offer ny explanation

In the non pretreated animals the [AChE] and [ChE] found at the time of death varied greatly According to the times of infusion the results

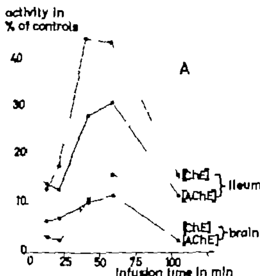
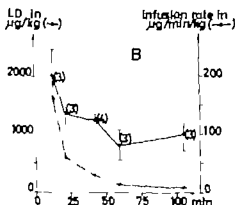


Fig 4. [AChE] and [ChE] in brai and ileum of guinea pigs given intravenous paroxan, correlated with the different rates of infusion used and the LD and the experimental time found.

The results of 16 experiments (taken from table 1) are divided in 5 gro ps according to the time of infusion. The mean values (see below) are shown, and the numbers of animals are indicated in brackets.

Part A Abscissa: Time of infusio in min. (For each group given as mean value).
Ordinate For each of the groups are indicated the mean values of [AChE] and [ChE] in the brain and in the ileum, as percentages of the controls.



Part B Abscissa The same as in part A.
Ordinate Left side Mean of LD of the groups i µg/kg Right side Mean of the infusio rates (in µg/min expressed per kg body weight).
The s.m. values for the LD are given.

from these 16 experiments were divided into 5 groups. The mean values of the [AChE] and [ChE] in the brain and the ileum (as percentages of the controls) are given in fig. 4 A. Generally they were lowest in the brain.

In fig. 4 B the average courses of the infusion rates and the LD corre-

ponding to the 5 groups in fig. 4A are shown. The interpretation of the results (from fig. 4A & B) mav be as follows. At a *high rate of infusion* of paroxan a high dose is necessary quickly to achieve a lethal accumulation of ACh. As a consequence the average activities will be found low At low infusion rates a long time of contact between enzyme and inhibitor will result in low activity in spite of the much lower LD At a *medium rate of* infusion the enzyme activities were usually found to be higher With another inhibitor than paroxan involving no spontaneous reactivation and given at an extremely low infusion rate, animals may survive without any measurable [AChE] At the medium rates of infusion the resulting slight inhibition is sufficient to cause death.

It is thus possible to kill the animals with an anticholinesterase agent at various degrees of inhibition apparently dependent on the *rate* of the administering. It may be assumed that a toxic accumulation of ACh (if this really is the cause of the poisoning) takes some time.

As shown in fig. 1A & B urethane reduces the lethal dose of paroxan This action could be of a different nature. Urethane itself is a weak reversible inhibitor of cholinesterases. In the doses used urethane will always cause the animal's death after 1–2 days or more (see WITLAND 1915) If the action of urethane is related to the anaesthetic properties, caution should be taken in the use of anaesthetics when treating the convulsions of anticholinesterase poisoning.

The biological functions, followed during the experimental periods, showed that respiratory changes (rate of air flow volume per cycle and per minute and the frequency) began about simultaneously with the infu sion Just after the infusion began a measurable degree of inhibition was hardly to be found Even a slightly reduced cholinesterase activity must accordingly have been able to produce changes in the biological functions (see fig. 2 and 3) cf JENSEN-HOLM (1965b) In fig. 3 three experiments involving paroxan infusion with simultaneous determination of E.C.G are illustrated The products of the time from P to R and P to P show that during the first two thirds only a slight increase took place and then a marked increase At the end of this last increase a total heart block was seen. This increase therefore would be valid indication of a serious development of poisoning.

In the ileum the intraluminal pressure was increased after the first fourth of the whole period showing increasing peristaltic contractions at increased frequency In the last fourth of the period these effects gradually stopped

In general, the different biological changes after infusion of paroxan at various rates took place after the same course of time (expressed as a percentage of the total time of infusion until death)

Summary

In urethane anaesthesia guinea pigs were killed by paroxan (diethyl-*p*-nitrophenyl phosphate, E-600) administered intravenously at different rates of infusion. Titrimetric determinations of [AChE] and [ChE] in the brain and the ileum were made. Different biological functions (respiration, blood-pressure and pulse rate, E.C.G., intraluminal pressure in the ileum) were followed in some of the experiments, usually by means of electronic equipment.

At increasing rates of infusion of paroxan the LD was increased. Maximally it was found to be 2.86 mg/kg at the highest rate and minimally 0.41 mg/kg as the average for the two lowest rates of infusion.

In guinea pigs pretreated with various doses of paroxan during the days before a constant LD was found, it being slightly lower than for the non-pretreated guinea pigs killed by the corresponding rates of infusion.

[AChE] in the brain was found to range between 1 and 22% and in the ileum from 1 and 54% of the corresponding control values. The averages were about 8 and 21% respectively.

[ChE] in the brain ranged between zero and 29% and in the ileum from 2 to 65% of the corresponding control activities. The averages were about 8 and 28% respectively.

Generally at high and low rates of infusion [AChE] and [ChE] contents were found to be much lower than after medium rates.

At the various rates of infusion used the biological changes described seem to take place after the same time (expressed as a percentage of the experimental period).

It is concluded that urethane if possible should be avoided in experiments into the lethal action of anticholinesterase agents, because urethane reduces the lethal doses of paroxan.

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Salicylates and Urinary Excretion of Histamine

By

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During a study of urinary excretion of histamine in patients with vascular headache (SJAASTAD & SJAASTAD 1964), we ran across a puzzling phenomenon.

A female patient, 46 years old (case no. I) during recurring bouts of Horton's headache, had found that magnyl ®[1]) was the only drug from which she benefited. Extensive use of magnyl impeded the interpretation of the histamine levels in the urine. Hence, she agreed to refrain from taking the drug during certain periods of investigation.

Whenever magnyl was withdrawn, an increase in histamine output seemed to take place. Conversely, readministration of magnyl, usually 8–12 tablets per day resulted in a clear lowering in urinary histamine excretion (SJAASTAD & SJAASTAD unpublished results)

The question then arose whether this was a general response to salicylate administration or a special effect of salicylates in a patient during attacks of headache.

Materials and Methods

Protocols

The 24 hour urinary histamine excretion was studied in 15 healthy adults, 6 female and 9 male, 26 to 52 years of age, before and after administration of magnyl.

Altogether 8 pills, two at a time, were given at intervals of 4–5 hours, and we performed 35 and 68 determinations, with and without medication, respectively. On 3 occasions the interval between a salicylate study and a repeated salicylate study or a study of normal excretion was 1–2 weeks. Otherwise the interval was around 3 weeks. In order to avoid systematic errors, studies with medication were interposed between studies without.

[1]) Magnyl ® each tablet consists of acetylsalicylic acid 0.45 g and magnesium oxide 0.05 g

The patient with Horton's headache mentioned bove and 1 patient with classical migraine were given the same dosage of magnyl in the free intervL

Since magnyl consisted of two components (i.e. acetylsalicylic acid and magnesium oxide), it was essential to examine the effect of each component Thus, 7 normal individuals were given 3.6 g of acetylsalicylic acid orally (0.9 g × 4) and 5 normal individuals 1 g of magnesium oxide (in one dose). Both components were admi histered as powder

Altogether 6 investigations on 4 individuals were performed with salicylic acid (0.8 g 4) and salicylamid (0.8 g × 4).

No other medication was allowed during collection of the urine. No dietary restrictions were imposed on the s bjects studied. For many however the diet was recorded. Usually the one of us who performed the analysis did not know whether drugs had been given or not to the patient on that occasion.

Methods for determination of histamine

The urine was passed directly into a 1 litre plastic flasks, containing 50–60 ml f 1.2 N hydrochloric acid, which produced a pH ∓ 3.5. During collection the flasks were stored at refrigerator temperature.

From 100 ml portions of urine histamine was extracted withi 2 3 hours of completing the collection. The histamine was adsorbed on n Amberlit IRC 50 cation-exchange column, as described by DUNÉR & PERNOW (1956). This method was slightly modified (O V SJAAST D, unpublished work). Histamine was eluted with hydrochloric acid, and the eluates were stored at +4 not longer than 2 3 days before assay on isolated tropinized guinea pig ileum. Two parallel tests were run on each specimen of urine. The values gi en represent thei means and are calculated as the base.

Conjugated histamine, i. e. the increment in free histamine after acid hydrolysis of the urine wa determined on a few samples. The recovery of histamine added to urine as measured t approximately week inters l during the study Mea recoveries of histamine acid phosphate and N-acetyl-histamine were 72 % (usually between 60 and 80) and 74 % respectively (O SJAASTAD, unpublished results). N corrections were made for histamine lost during the extraction.

Results

Studies with magnyl

The results of magnyl studies on healthy individuals are summarized in table 1 The mean excretions with and without magnyl were 7 6 ± 1.3 and 15 1 ± 1 4 (s.e.m.) μg/24 hours, respectively ($P < 0.01$ T test null hypothesis, $P < 0.005$) In 14 of 15 subjects a decrease in the average output of histamine took place In three individuals, the decrease was negligible. Of fourteen patients studied at least 4 times 6 showed no overlapping.

Assay with an "internal standard" did not indicate the presence of inhibitory substances

In two subjects (L.G and T O in table 1) magnyl was given on 3 consecutive days, the histamine output being determined on the first and last day On both the excretion of histamine remained low

Table I

Influence of magnyl on the urinary excretion of histamine.

	No. of observations	Urinary excretion in µg/24 hours Average	Range	
R.T.R.	a 3	18.7	12.9–24.5	
	b 3	4.3	3.8– 5.1	
P.T.R.	a 1	13.3	13.3	
	b 3	4.0	1.8–10.2	
A.S.	a 2	15.4	11.6–19.2	
	b 2	19.1	18.6–19.7	
L.G.	a 5	13.6	5.5–22.4	
	b 2	5.7	5.5– 5.9	(5.9–4.3)
K.F.	a 4	14.9	8.0–25.4	
	b	2.7	2.5– 2.8	
A.R.	a 4	7.9	5.4–10.8	
	b 2	6.1	5.8 6.3	
G.R.	a 4	17.3	8.6–27.3	
	b 2	9.7	9.1 10..	
I.F.	a 4	11.8	10.8–12.7	
	b 1	2.5	2.5	
A.P.	a 1	8.9	8.9	
	b 1	8.7	8.7	
I.H.	a 4	17.1	11.6–21.4	
	b 1	3.3	3.3	
O.S.	a 8	15.7	9.3–22.6	
	b 3	10.0	6.6–16.5	
T.H.	3	11.6	6.9–15.9	
	b	2.6	1.9– 3.3	
T.O.	a 8	11.0	6.0–20.3	
	b 4	10.9	2.4–36.0	(2.4–2.9)
H.H.	a 10	19.0	11.2–27.9	
	b 4	11.7	7.7–16.9	
L.R.	7	29.7	16.0–48.3	
	b 3	13.1	<3.8–26.3	

Mean without medication 15.1 ± 1.4 µg/24 hours
Mean with medication 7.6 ± 1.3 µg/24 hours

magnyl = acetylsalicylic acid 0.45 g and magnesium oxide 0.05 g. 8 tablets were given during each study

= without the drug.

b = with the drug.

The values in brackets of L.G. and T.O. represent the excretion on day 1 and 3 of a 3 days experiment with magnyl.

Table 2

Effect of magnyl in two patients with headache during free interval.

	N of observations	Urinary excretion in µg/24 hours	
		Average	Range
M.S	1	7.8	7.8
	b 1	13.0	13.0
E.U	7	8.9	1.4–19.0
	b 3	37.5	7.9–56.8

a = without the drug.
b = with the drug.

During the free interval, both patients with vascular headache seemed to react in a way contrary to the usual and to what was originally observed during attacks of headache in one of them. (E.U) (table 2).

Magnyl was administered to two other patients during their headaches. The effect of this seemed to be small and the phenomenon originally observed was therefore not studied more closely

Studies with acetylsalicylic acid

The average excretion with medication was 15.2 µg/24 hours (see table 3)

Generally the pattern of reaction after administration seemed to be similar to that observed after magnyl One subject, P T R., however showed a clear increase in output after acetylsalicylic acid On the other hand he showed a clear decrease in histamine output after magnyl. This suggests that the increase observed is not due to acetylsalicylic acid itself Nevertheless, the possibility that an altered mode of reaction to acetylsalicylic acid was present in this particular individual at the time of study remains open However most likely this elevation in histamine excretion is analogous to what may be encountered spontaneously in healthy individuals on rare occassions. If this is so it will be of interest to compare the output observed in the six remaining subjects before and after medication.

The mean excretion after acetylsalicylic acid was 9.2 ± 2.2 µg/24 hours. This value is significantly different from the control excretion by all the subjects, i.e 15.1 ± 1.4 µg/24 hours, ($P < 0.05$) However if the control values of only the same 6 individuals are used, which is a more logical approach, a mean of 13.8 ± 1.8 µg/24 hours is found ($P > 0.05$.

Table 3

Effect of 3.6 g of acetylsalicylic acid on urinary excretion of histamine.

	No. of observations	Urinary excretion in µg/24 hours	
		Average	Range
I F	a 4	11.8	10.8–12.7
	b 2	9.0	6.4–11.6
K.F	4	14.9	8.0–25.4
	b	5.7	4.5–6.8
T.H.	a 3	11.6	6.9–19.8
	b 1	5.[illegible]	5.2
G.R.	4	17.3	8.6–27.3
	b 3	13.[illegible]	6.0–19.1
A.R.	4	7.9	5.4–10.8
	b 1	4.9	4.9
H.H	a 10	19.0	11.[illegible]–27.9
	b 2	16.9	18.5–15.2
P.T.R.	a 1	13.3	13.3
	b 1	51.8	51.8

Mean without medication 13.8 ± 1.8 µg/24 hours
Mean with medication 9.2 ± [illegible].2 µg/24 hours

The means represent only the 6 first mentioned individuals.
a = without the drug.
b = with the drug.

T-test). The average decrease on the other hand was found to differ significantly from zero ($P < 0.05$). The average excretion after magnyl was 5.9 µg/24 hours.

Studies with salicylamide, salicylic acid and magnesium oxide

The mean excretion after salicylates was 9.7 µg/24 hours and without them 16.2 µg/24 hours. The average decrease in excretion differed significantly from zero ($P < 0.05$).

The mean excretions with or without 1 g of magnesium oxide were both 13.1 µg/24 hours (see table 4). The dosage of magnesium oxide was 2.5 times the dosages given during a magnyl study.

Effect of salicylates on urinary contents of conjugated histamine

In altogether 5 individuals magnyl, acetylsalicylic acid and salicylic acid were found to be without any clear effect on the amount of urinary conjugated histamine.

Table 4

Effect of 1 g of oral magnesium oxide on urinary excretion of histamine.

	No. of observations	Urinary excretion in μg/24 hours	
		Average	Range
T H	a 4	13.7	6.9–19.8
	b 2	8.6	7.1–10.1
O V.S.	a 3	11.0	8.4–14.3
	b 3	16.3	12.0–21.8
O.S.	8	15.7	9.3–22.6
	b 3	12.4	10.5–13.9
H H	10	19.0	11.2–[illegible]7.9
	b 2	22.2	15.8–[illegible]8.6
R.A.	a 3	8.4	8.0–8.8
	b 1	6.0	6.0

Mean, with or without medication 13.1 μg/24 hour

a = without the drug.
b = with the drug.

Discussion

Low urinary excretion of histamine in man has previously been reported in e.g., bronchial asthma (DUNÉR & PERNOW 1958) food allergy (DUNÉR & PERNOW 1960) ecclampsia gravidarum (BJURO *et al.* 1961), and multiple sclerosis (O. SJAASTAD in press). Besides, in the rat, low excretion has been observed after adrenalectomy (BJURO 1963) or after administration of anabolic steroids (KIM 1961, WESTLING & WETTERQVIST 1962).

In healthy individuals salicylates also seem to produce a lowering of urinary histamine. A tendency towards low urinary excretion was encountered with all salicylates studied. In this material, however, the decrease was most pronounced after the administration of magnyl.

The only difference between the dose of acetylsalicylic acid and magnyl administered is 0.4 g of magnesium oxide. It is worthy of note that in the rat a magnesium-deficient diet produces mast cell disruption (BÉLANGER *et al.* 1957) and increased urinary levels of histamine (BOIS *et al.* 1963, WEST 1964). Even 1 g of magnesium oxide did not produce any opposite effect on the urinary excretion of free histamine. The extra ingestion of magnesium oxide does, therefore, hardly in itself explain the more pronounced effect of magnyl. Thus the observed effect of magnyl seems to be caused by the acetylsalicylic acid component.

No valid explanation for the apparent difference in potency of mag-

nyl and acetylsalicylic acid in lowering urinary free histamine [illegible] offered.

In the experiments recorded here the coefficient of [illegible] for urinary histamine was 8 7/ No marked fluctuations [illegible] were observed. It thus seems unlikely that the results can arise from methodological reasons during assay At this point, however [illegible] tioned that this method does not give such accurate results [illegible] high levels of histamine (for discussion, see O SJAASTAD [illegible]

However other factors could be of importance for the [illegible] the results. Conceivably differences in salicylate concentration [illegible] body due to different urinary acidity might to some [illegible] inter-individual variations in response. The acidity of the [illegible] *could not be checked because of the experimental* [illegible] from a mixed diet, as in the present series, a considerable [illegible] variation in histamine excretion is encountered (O S [illegible] lished)

Theoretically salicylates may reduce histamine [illegible] several mechanisms.

1 Salicylates at low concentrations are known [illegible] (PARROT & LABORDE 1961) and glutamate (B[illegible] carboxylase from the rat *in vitro*

2. TRETHEWIE (1959) found that sodium salicylate [illegible] acid caused inhibition of release of histamine [illegible] anaphylactic lungs. Salicylate may thus have an [illegible] of histamine from cells

More remote possibilities also exist

3 Altered metabolism of histamine might [illegible] same finding. Conjugated histamine was [illegible] this series. No specific trend was observed. A [illegible] ever hardly be based on the measurement of [illegible] this substance probably does not reflect the [illegible] histamine solely (DUNÉR & PERNOW 1960 [illegible]

4 The doses used in the present [illegible] anorexia. MITCHELL & CODE (1954) observed [illegible] lower excretion of histamine than did [illegible] does, however not seem very likely

Another observation may elucidate the [illegible] urinary histamine (BROGREN *et al* 1958[illegible] dose of acetylsalicylic acid a patient [illegible] a severe anaphylactic reaction [illegible] urinary output of histamine. [illegible] a marked [illegible]

During a free interval our patients [illegible]

same kind of response as to the urinary output of histamine. In a few instances normal individuals also showed increases in urinary histamine output, which might be secondary to the administration of salicylates. The possibility therefore exists that there are two opposite modes of response to salicylate administration in so far as it involves the urinary excretion of histamine.

Summary

Salicylates were administered orally to 16 healthy adults. A significant decrease in urinary excretion of histamine was observed after all salicylates studied. The most significant decrease in excretion was, however observed after magnyl (one tablet 0 45 g acetylsalicylic acid and 0 05 g magnesium oxide). As magnesium oxide did not affect the urinary excretion of histamine, it is concluded that the most likely active component is acetylsalicylic acid

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BRITISH PHARMACOLOGICAL SOCIETY

MEMORIAL TO J H GADDUM

The British Pharmacological Society would like to honour the memory of J H Gaddum in a manner which will advance the science of pharmacology The memorial might take the form of an occasional award to a younger scientist from any part of the world who has made an important contribution to the field of pharmacology or a closely related subject The award might consist of an invited lecture to be arranged by the British Pharmacological Society as well as a prize and provision for travelling expenses

The Society has instructed us to invite contributions which may take the form either of a single donation or for those U K. residents who wish it a seven-year covenant Such contributions should be made payable to the J H. Gaddum Memorial Fund and addressed to the Treasurer of the British Pharmacological Society Professor D R Wood, Pharmacology Department, School of Medicine Leeds 2, England It is intended that the fund shall be administered by trustees and held solely for charitable purposes as outlined above We would be grateful if contributions or promises could be sent as soon as possible and not later than August 31st 1966

G A H. Buttle M. V
March 196 J R. Vane D

same kind of response as to the urinary output of histamine. In a few instances normal individuals also showed increases in urinary histamine output, which might be secondary to the administration of salicylates. The possibility therefore exists that there are two opposite modes of response to salicylate administration in so far as it involves the urinary excretion of histamine

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Accelerated Uptake of Mercury by Brain caused by 2,3-Dimercaptopropanol (BAL) After Injection into the Mouse of a Methylmercuric Compound

By

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In a previous paper (BERLIN & ULLBERG 1963) we have shown that the body distribution of mercury in the mouse after injection of an alkyl mercury compound (methylmercuric dicyandiamide) differs from that found after injection of either an inorganic (mercuric chloride) or an aryl (phenylmercuric acetate) compound of mercury It has also been shown that dimercaprol(=BAL=(=2,3-dimercaptopropanol) causes an increased uptake of mercury in the brain when given to mice exposed to inorganic or phenylmercuric compounds (BERLIN & LEWANDER 1965 BERLIN & RYLANDER 1964) at the same time, dimercaprol considerably decreases the acute mercury load in the kidney In chronic poisoning due to methylmercuric compounds, the dominant toxic signs are due to disturbed function of the central nervous system (HUNTER *et al* 1940). As AHLMARK & AHLBORG (1949) have reported favourable clinical results from dimercaprol therapy in some cases of chronic methylmercuric poisoning, it was considered useful to study the effect of dimercaprol on mercury distribution in mice exposed to a methylmercuric compound, especially in the central nervous system, where it has already been shown that mercury given in this form accumulates in considerable amounts (BERLIN & ULLBERG 1963). In the first part of our inquiry therefore, the mercury distribution found in mice given methylmercuric dicyandiamide alone was compared with that found in others given methylmercuric dicyandiamide together with dimercaprol The comparison was made by autoradiography of thin sagittal whole-body sections of animals killed at various times after exposure. In the second

part of the study two groups were given repeated daily doses of methylmercury. One group was also given dimercaprol. The body burden of mercury was measured daily during the whole experiment, and after 16 days the animals were killed and organ-assays were performed.

Methods

Single exposure

Two groups of 5 mice of the CBA strain, obtained from the Genetic Institution of the University of Stockholm, were given via a tail vein, ^{203}Hg-labelled methylmercuric dicyandiamide in a dose corresponding to 0.5 mg Hg/kg body weight. The methylmercuric dicyandiamide was synthesized as described previously (BERLIN 1963) and had a specific activity of 1.5 C/g Hg. One group was also given dimercaprol in a dose corresponding to 0.3 mg dimercaprol/kg body weight together with the mercurial. One control- and one dimercaprol-treated animal were then killed 1 hour, 1, 4, 8 or 16 days after injection, by immersion, under ether anaesthesia, in a mixture of solid carbon dioxide and acetone (−80°). Sagittal sections, 20 μ thick, were cut at −10° by the method described by ULLBERG (1958). Each section, affixed to cellulose tape, was apposed to x-ray film together with an isotope reference scale consisting of 14 steps of decreasing isotope concentration, the ratio between steps being 1 : 2. Section and film were stored at −10° until exposure was sufficient to give an autoradiogram of the mercury distribution in the section. The order of mercury concentration in different tissues and organs was then determined by comparing the darkening caused by the tissues and that caused by the material in the reference scale (BERLIN & ULLBERG 1963).

Prolonged exposure

Thirty male mice (20–25 g) of the CBA strain were randomized into two groups of 15 mice each. Each day for 16 days both groups were injected intraperitoneally with a solution of radioactive methylmercuric dicyandiamide. The animals received a dose corresponding to 0.05 mg Hg/kg body weight each day. The mercury used had a specific activity of 0.5 C/g. One group was also given intramuscular injections of dimercaprol (2 mg/kg body weight) dissolved in arachis oil. The mice were kept in four cages, the same number of animals from both groups in each cage. The measurements of the body burden of mercury were performed by scintillation counting with a NaI crystal (as described by BERLIN & LEW[illegible] 1965). After 16 days of exposure the animals were killed by prolonged ether anaesthesia. The brain, lung, liver, kidneys, testes, myocardium and a piece of striated muscle were removed and dissolved in concentrated hydrochloric acid to obtain equal efficiency for all organs by the measurement. Scintillation counting in a well crystal was then performed on the solutions, with at least 10,000 counts each time. The background was about 1,000 counts per minute, and none of the counts fell below twice the background value.

Results

Autoradiographic findings after single exposure

The autoradiograms show that dimercaprol induced a significant change in the behavior of mercury in the body after injection of methyl mercuric dicyandiamide. When the mercurial alone is given, mercury

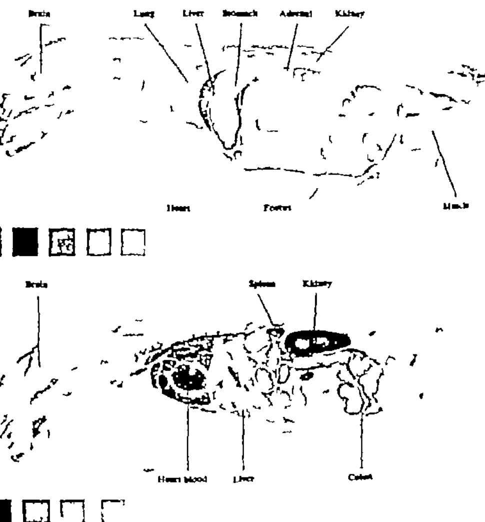


Fig. 1 Autoradiograms of sagittal whole-body sections of mice killed 1 hour after injection of methyl ^{203}Hg dicyandiamide dimercaprol (BAL) (upper) and methyl ^{203}Hg dicyandiamide (lower) The upper section is from pregnant mouse. The isotope reference scale accompanying each section is shown the activity ratio between adjacent steps is 1 2.

tends to persist in the blood and leaves slowly during the first 24 hours. When dimercaprol is also given, mercury disappears rapidly from the blood and is distributed within 1 hour through the body in a pattern that appears only after 8 days when methylmercuric dicyandiamide alone has been injected Fig. 1 shows the mercury distribution one hour after injection in the two circumstances the differences in distribution are clear from the autoradiograms.

As found previously (BERLIN & ULLBERG 1963) mercury given as a methylmercuric salt accumulates in the brain only slowly in control

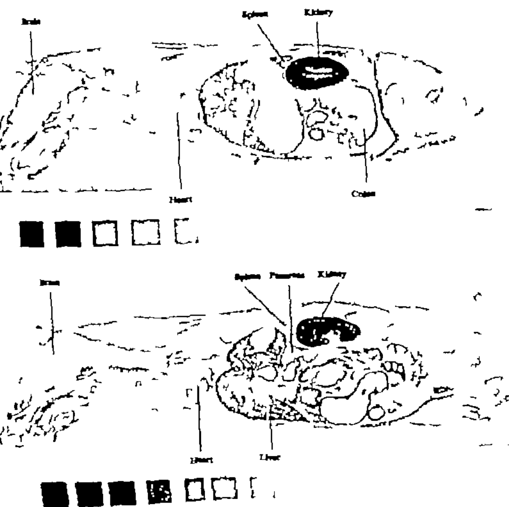


Fig. 2. Autoradiograms of sagittal whole-body sections of mice killed 24 hours after injection of methyl ^{203}Hg dicyandiamide + dimercaprol (BAL) (upper) and methyl ^{203}Hg dicyandiamide (lower). The isotope reference scale accompanying each section is shown; the activity ratio between adjacent steps is 1:2.

animals, and maximum concentration is not reached until 8 days after injection. In the dimercaprol-treated animals, however, mercury appears early in the brain and reaches within 24 hours the same maximum concentration as is seen only after 8 or 16 days in the controls. Fig. 2 illustrates the difference between mercury accumulation in the brain of a control animal and of a dimercaprol-treated animal 1 day after injection. At 8 and 16 days after injection mercury distribution in the treated animals is almost identical, as evidenced by autoradiograms

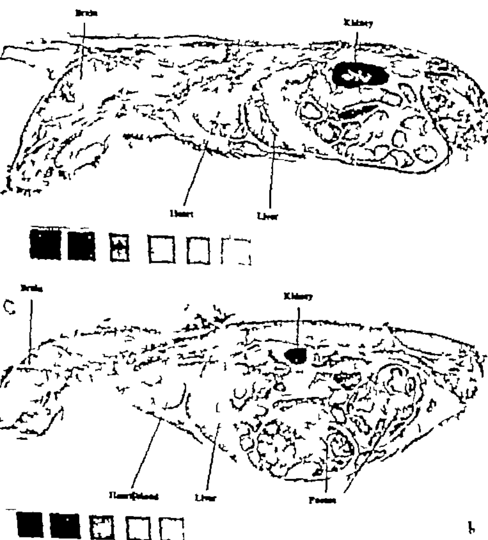


Fig. 3 Autoradiograms of sagittal whole-body sections of mice killed 8 days after injection of methyl ^{203}Hg dicyandiamide + dimercaprol (BAL) (upper) and methyl ^{203}Hg dicyandiamide (lower). The isotope reference scale accompanying each section is shown; the activity ratio between adjacent steps is 1:2.

that in the controls (fig. 3) It should be pointed out, however that a minimum difference in mercury concentration of about two fold is necessary for a change to be detected in the autoradiograms Fig. 4 shows in tabular form results of densitometric measurements of the orders of highest concentration in some of the organs, at three intervals after the injection. The autoradiograms do not indicate any increased mercury elimination after dimercaprol treatment.

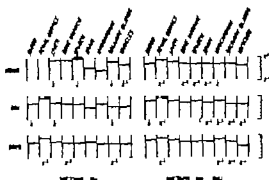


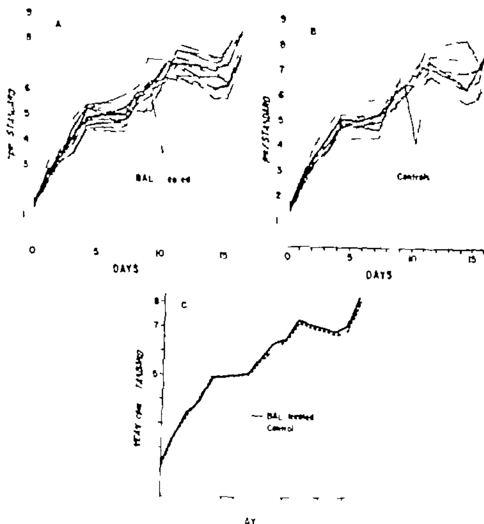
Fig. 4. A comparison between the distribution of mercury among different organs at 3 different times after injection of methyl 203Hg dicyandiamide with and without dimercaprol. The highest concentration in each organ measured densitometrically from an autoradiogram, is shown as a vertical bar against geometric scale (common ratio ½), and is also given in arbitrary units as a power of 2.

Whole-body counting and organ assay after repeated exposure

During the 16 days of exposure no significant differences could be shown between the dimercaprol-treated and control groups by whole body counting (fig. 5a, b & c). Nor did organ assay reveal any statistically significant differences between the animals in the two groups (t-test at the 5 / level (table 1).

Discussion

The experiments show clearly that, when dimercaprol is injected together with methylmercuric dicyandiamide, mercury penetrates more rapidly into all tissues than when the mercurial is given alone. In the central nervous system the increase of mercury inflow is especially pronounced These results may be useful to apply to studying the mechanism of mercury uptake in the brain, which is not very well understood. To what extent the methylmercuric radical is combined in treated animals with dimercaprol is not revealed by the present investigation However it is difficult to explain the redistribution of mercury caused by dimercaprol without assuming that the methylmercuric radical exists for some time in the form of a methylmercuric dimercaprol complex. It is probable that this complex, after penetration into tissue, is split and that the methylmercuric radical is taken up by competing ligands. On the other hand, it is also difficult to account for the favourable clinical effect of dimercaprol treatment in methylmercuric poisoning along with the results of our work without assuming that the methylmercuric radical in the brain forms a stable complex with dimercaprol that is less toxic than the radical



F g 3 Consecutive total body scintillation count of mice ( ) after methyl 203Hg dicyandiamide dimercaprol (BAL) (b) fter methyl 203Hg dicyandiamide. Each thin line represents result from ngle nimal (c) mean of each of the tw groups.

alone It may however be justifiable to conclude from our results that dimercaprol forms a complex with the methylmercuric radical which penetrates the blood-brain barrier more rapidly than the methyl mercurial otherwise does If BAL truly alleviates the effects of methylmercuric poisoning, in which event the alkylmercury is accumulated in the brain, it seems reasonable to assume that dimercaprol can penetrate into the brain and there form a complex with the alkylmercury radical so facilitating the movement of the methylmercuric radical out of the brain The results presented here clearly warrant further inquiry into the effect

Table 1

^{203}Hg retained after 16 days in some organs of the dimercaprol (BAL)-treated animals and the controls.

		BAL + Hg		Hg	
		Mean	Range	Mean	Range
Brain	Organ weight, g	0.404	0.455–0.381	0.400	0.432–0.346
	Activity cpm	11 020	14,340–8,228	9,976	12,330–6,991
	cpm/g	27,268	33,580–20,216	25,056	32,110–17 790
Lung	Organ weight, g	0.086	0.123–0.045	0.088	0.148–0.028
	Activity cpm	5,143	7,983–2,828	5,019	7,387 2,426
	cpm/g	60,306	86,220–47,880	61 116	86,650–27,060
Liver	Organ weight, g	0 614	1 039–0.316	0.675	1.057–0.391
	Activity cpm	60,890	119,500–36,830	61 169	90,800–35,680
	cpm/g	89,089	107 400–63,350	89 706	127,820–53,220
Kidney	Organ weight, g	0.198	0.230–0.175	0.202	0.227–0.174
	Activity cpm	60,461	70,480–40,100	64 898	80,266–56,425
	cpm/g	305,283	378,100–219 130	3 7,800	413,200–268,700
Testis	Organ weight, g	0.074	0.091–0.053	0.075	0.094–0.065
	Activity cpm	2,102	2,771–1 452	[illegible],129	2,830–1 407
	cpm/g	28,866	35,990–19,200	28,794	39,860–20,860
Myocardium	Organ weight, g	0.093	0.112–0.070	0.097	0.116–0.084
	Activity cpm	5 559	7,054–3,885	5,654	7 650–3,599
	cpm/g	58,882	80,470–44,270	58,648	81,380–35,990
Muscle	Organ weight, g	0.220	0.350–0.121	0.225	0.324–0.146
	Activity cpm	12,340	29,360–8,045	12,555	18,950–8,340
	cpm/g	57 479	97,340–43,530	56,489	74,750–38,760

of dimercaprol in methylmercuric poisoning, before dimercaprol can be recommended for the therapeutic use, especially in acute methylmercuric poisoning. The results also emphasize the need for further knowledge of the bodily distribution and metabolism of the dimercaprol molecule. Earlier investigations (TAMBOLINE *et al* 1955) have shown that dimer caprol is largely detoxified and eliminated within 48 hours after injection. However it still remains possible that dimercaprol persists unchanged for a longer time in lesser amounts in some tissues, for example in the brain.

Summary

The effect of intravenously injected dimercaprol (0.3 mg/kg) on the body distribution of mercury in mice killed at various times up to 16 days after a single dose of methyl ^{203}Hg dicyandiamide (0.5 Hg/kg) was studied by autoradiography of sagittal whole body sections. It was shown that dimercaprol accelerated the distribution of mercury from blood into tissues the 1 hour distribution in dimercaprol treated animals was similar to the 4-day pattern in control animals. The brain uptake of mercury in dimercaprol treated mice was particularly accelerated

No detectable increase in mercury elimination (measured by body scintillation counting and organ assay) was noted in 15 animals given intramuscular injections of dimercaprol (2 mg/kg body weight) when compared with 15 control animals. Both groups had received daily injections of methyl ^{203}Hg dicyandiamide.

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Clearance Experiments on the Effect of Probenecid on Urate Excretion in the Rabbit

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It is now generally accepted that the excretion of urate in man, dog, and rabbit occurs by a three fold mechanism, involving glomerular filtration, tubular reabsorption and secretion of urate (GUTMAN YÜ & BERGER 1959 POULSEN & PRÆTORIUS 1954 LATHEM, DAVIS & RODNAN 1960) In the rabbit, reabsorption of urate is found at low plasma levels (5–20 µmol/l), but net secretion is readily demonstrated during infusion of urate (POULSEN & PRÆTORIUS 1954) or osmotic diuretics (MØLLER 1962) Studies carried out by YÜ BERGER, KUPFER & SHERMAN (1960) similarly show the existence of a distinct secretion in the Dalmation dog. In contrast, urate secretion in man (GUTMAN, YÜ & BERGER 1959) and mongrel dog (YÜ BERGER, KUPFER & SHERMAN 1960) is only detectable under extreme experimental conditions, comprising heavy loading with mannitol, urate infusion and administration of uricosuric drugs in order to inhibit tubular reabsorption of urate.

It is of interest that probenecid diminishes the renal excretion of urate at high plasma urate concentrations in the rabbit (POULSEN 1955) After intravenous injection of 50–150 mg probenecid per kg bodyweight, a urate-creatinine clearance ratio (C_{Ur}/C_{Cr}) of 0 8–0 9 was observed It was pointed out by POULSEN (1955) that probenecid in appropriate dosage *may completely arrest the secretion of urate, but the possibility exists* that both secretion and reabsorption of urate are inhibited in such a manner that nearly equal amounts of urate are transported by secretion and reabsorption after administration of probenecid.

The purpose of the experiments recorded here was to provide more detailed information about the effect of probenecid on urate excretion in the rabbit. The effect of probenecid on urate excretion was studied during infusion of saline and mannitol. The findings are in accordance with the view that probenecid is able almost completely to block the secretion of urate.

Methods

The experiments were conducted on male rabbits, weighing between and 4 kg, fed on a diet of turnips and hay Food was withdrawn 4 hr befor the beginning of an experiment, but the animals had free access to water

Animal experiments

At the beginning of an experiment, 70 mg pentobarbital sodium (nembutal ® Abbott) per kg bodyweight were injected intravenously and a T-tube was inserted in the trachea under local anaesthesia (lidocain, 0.5 %). One branch of the T-tube was connected with a respiration pump giving 45 inhalations/min., and the animal was put under surgical anaesthesia by a further injection of 25 mg pentobarbital sodium/kg bodyweight.

The internal jugular vein and carotid artery were dissected free on one side and cannulated with polyethylene tubing. The infusion fluid contained creatinine or inulin or both at a concentration of 0.5 (w/v) in physiological saline. In most experiments the infusion fluid contained urate at a concentration of 0.08–0.1 % (w/v), and in some mannitol was added to the infusion fluid t induce osmotic diuresis. Stock solutions of urate were made by neutralizing 1 g of uric acid with 0.3 g of lithium carbonate in 100 ml boiling water The infusion rate was 3 ml/min.

When the infusion had ru for about 1 hr urine collections were started from a urethral catheter Urine was collected for 3–5 periods for control determination of urate excretion. After infusion of 100 mg probenecid (uricosid ®)[1] per kg bodyweight over a period of 20 min. or induction of osmotic diuresis by infusion of mannitol the urate excretion was followed for another 3–5 periods. The clearance periods had duration f 10 min. each (except during vigorous osmotic diuresis, when the clearance periods lasted 5 min.). At the end of each period the bladder was washed twice with 5 ml water Blood samples were drawn from the arterial cannula and delivered into centrif ge tubes containing heparin. The first 8 drops f blood were discarded, to be sure of obtaining a sample fully representative of circulating arterial blood. The blood was taken 2 or 3 min. before the middle of the clearance periods, as a correction for the time it takes urine to travel from the glomerular membrane to the bladder (2 min. being used when the diuresis was larger than 2 ml/min.).

Analytical methods

Urate i urine and plasma was measured by enzymatic spectrophotometry (PRAETORIUS & POULSEN 1953) small concentrations f urat were assayed on trichloracetic acid filtrates by the method previously described (MØLLER 1962). Creatinine was determined by the method of OWEN, IGGO, SC NDRETT & STEWART (1954), but omitting adsorption on Fuller's earth. I ulin in plasma and urine was determined as described by SCHREINER, 1950 the glucose contents f plasma samples were determined by the method of H GEDORN, HALLSTRÖM & NO MA JENSEN (1946) i order to correct for the colour development caused by glucose.

Ultrafiltration experiments

The pparatus of TORIBARA, TEREPKA & DEWEY (1957) was used t examine the ultrafiltr bility f urate f om rabbit plasma. Quantities of 3 ml plasma were placed in a cellophane bag (Visking Nojax C il g R) and centrifuged at 1 9 × 10[3] g. The concentration of urate in ultrafiltrates of plasma eraged 103 % of the plasma concentration (5 bnals,

[1]) Pr benecid was kindly upplied by Alfred Benzon, Ltd.

range 101 106%). Under our experimental conditions, the plasma protein concentration was about 5–6%. According to SVEDBERG & SJÖGREN (1928), plasma proteins have a specific volume of 0.75 ... the basis of these figures the volume of the plasma proteins may be estimated as approximately 4%. This means that the concentration of urate in the ultrafiltrate o... an average was 99% ...f the concentration in the plasma w...ter indicating complete ultrafiltrability of urat... from rabbit plasma.

Comparisons between creatinine and inulin clearance

It has been assumed for many years that the clearance of creatinine (C_{Cr}) ... the dog and rabbit is identical with the glomerular filtration rate (for a discussio... of this subject, see SMITH 1951). More recent experiments by the stop-flow method hav... revealed that creatinine is secreted in the proximal tubule of the dog and that the excretion of creatinine may be influenced by various drugs (O'CONNELL, ROMERO & MUDGE 1962).

KAPLAN & SMITH (1935) have demonstrated equality between C_{Cr} and inulin clearance (C_{In}) at different plasma concentrations of creatinine in the rabbit. In our study comparisons have been made between C_{Cr} and C... under various conditions. During infusion of physiological saline, the ...verage value of C_{Cr}/C_{In} was 1 01 (5 experiments, ra...ge 0.98–1.03). In other experiments, the clearance values were compared during infusion of mannitol. Here, a mean value of 1 03 (6 experiments, range 0.98–1 11) was obtained. Finally 6 experiments were carried out after ...ntravenous administration of 100 mg probenecid/kg body-weight. I... these C_{Cr} was always somewhat smaller than C_{In}, C_{Cr}/C_{In} ...veraging 0.92 (range 0.88–0.95). This value is significantly different from the values obtained during infusion of physiological saline ($P < 0.001$).

The results show only small differences between C_{Cr} and C_{In} in the experiments with infusion of physiological saline or mannitol. However ...dministration of probenecid leads to a minor decrease in C_{Cr} relative to C_{In}, indicating that C_{Cr} is presumably somewhat smaller than the glomerular filtration rate after administration of probenecid.

Results

Effect of probenecid on urate excretion during infusion of urate

The results of experiments in which probenecid has been administered during infusion of 1 6–1 8 mg urate/min are summarized in table 1 In these experiments, urate clearance (C_{Ur}) was compared with C_{Cr} The first 3 experiments were performed during infusion of physiological saline, but in experiments 4 5 and 6 mannitol was added to the infusion fluid at a concentration of 3–5 / (w/v) However no differences in the response to probenecid could be detected between the 2 groups of animals, and they will therefore be considered together

In the control periods, C_{Ur}/C_{Cr} ranged from 1 14 to 2.63 Infusion of probenecid resulted in a pronounced decline of urate excretion, C_{Ur}/C_{Cr} in all experiments, ranging from 0.84 to 0.94 under these conditions. The plasma urate concentration did not change markedly after administration of probenecid P_{Ur} rose from a mean of 168 µmol/l to 202 µmol/l.

A decrease in C_{Cr} was generally observed during the course of an

Table 1

Effect of probenecid on urate excretion (C_{u}/C_{cr}) during infusion of urate.

Experiment no.	Infusion of mannitol	Administration of probenecid	P_u (µM/l)	C_{cr} (ml/min.)	C_{ur}/C_{cr}	V/C_{cr}
1	0	0	[illegible]54 (238 [illegible]67)	13 1	1 91 (1 76–[illegible]03)	.21
		+	313 (308 317)	11.8	0.90 (.8 96)	.21
[illegible]	0	0	138 (134–140)	16.[illegible]	1.57 (1 46–1.67)	11
		+	199 (197 [illegible]00)	9 7	.84 (.80– 91)	14
3	0	0	167 (135–207)	14 9	1 14 (1.08 1.[illegible]1)	.07
		+	146 (136–167)	15.3	.87 (.82 90)	.07
4	+	0	128 (120–138)	11 9	2.63 ([illegible]56–[illegible]71)	18
		+	130 (11 146)	8.5	.85 (73–1 03)	12
5	+	0	96 (90–103)	1[illegible]1	1.81 (1 70–1.88)	[illegible]5
		+	127 (124–133)	8.[illegible]	.94 (86– 99)	[illegible]3
6	+	0	2[illegible]7 (221–238)	15 7	1 76 (1.69 1 77)	.35
		+	296 ([illegible]67 321)	10.2	91 (.85– 94)	.29

P_{cr} = plasma concentration of urate C_u and C_{cr} = clearance of urate and creatinine, respectively V = diuresis. The [illegible]alues given [illegible]re mea[illegible]s, with highest and lowest values in pa[illegible]entheses.

periment thus C_{cr} averaged 14 0 ml/min. before and 10 6 ml/min. after administration of probenecid.

The experiments so far reported confirm the results of POULSEN (1955) for the effect of probenecid on urate excretion. It is remarkable that, in spite of widely varying C_r C_{cr} ratios in the control periods, C_r/C_{cr} only ranged from 0 84 to 0 94 after infusion of probenecid.

Effect of severe osmotic diuresis on C_{Ur}/C_{In} after administration of probenecid

The results of two experiments (no 7 and 8) on the effect of vigorous osmotic diuresis (induced by infusion of 10% mannitol) are shown in table 2. Probenecid was infused before the collection of urine samples, and urate was infused during the whole experiment.

It can be seen from the table that mannitol infusion caused a rise in V/C_{In} from 0 17 and 0.14 to 0.34 and 0.52, respectively in the 2 experiments. In experiment no 7 the value of C_{Ur}/C_{In} was 0 84 both before and after infusion of mannitol, whereas a modest increase in C_{Ur}/C_{In} from 0 82 to 0 89 was observed in experiment no 8

The experiments show that after administration of probenecid net reabsorption of urate persists, even during vigorous diuresis

Effect of probenecid on urate excretion at low plasma concentrations of urate

POULSEN (1955) has reported that he was unable to detect a consistent effect of probenecid on urate excretion at low endogenous concentrations of plasma urate. In our study urate excretion was examined during infusion of mannitol and physiological saline, but without administration of urate The results are those for the last 5 experiments shown in table 2.

Mannitol was infused in experiments 9 and 10 In agreement with a previous study (MØLLER 1962) net secretion of urate was found in the control periods. Administration of probenecid led to a distinct decrease in urate excretion, the value of C_{Ur}/C_{In} now being 0.84 and 0 75 Further a rise in the plasma concentration of urate was noted.

The effect of probenecid during saline diuresis is shown in the last 3 experiments recorded in table 2. A fall in C_{Ur}/C_{In} was found in all 3 experiments. The value of C_{Ur}/C_{In} averaged 0 68 in the control periods, and 0 45 after infusion of probenecid that of P_{Ur} rose from a mean value of 9 4 µmol/l to 11 0 µmol/l.

Discussion

Experiments on the effect of probenecid on urate excretion are reported in this paper In agreement with POULSEN (1955), the value of C_{Ur}/C_{Cr} after administration of probenecid falls to about 0.8–0.9 during infusion of urate. As the results obtained by our ultrafiltration studies show that urate is not bound to the plasma proteins in the rabbit, and comparisons between C_{Cr} and C_{In} indicate that the glomerular filtration rate has probably been somewhat underestimated after infusion

Table 2

The effect f probenecid on urate excretion (C $_{u}$/C$_{In}$) at high and low plasma levels of urate.

Experiment no.	Infusion of mannitol	Administration of probenecid	No. of periods	P$_{u}$ (µMol/l)	C (ml/min.)	C$_{u}$/C$_{In}$	V/C
7	0	+	3	324 (317 334)	10.8	.84 (.78 .87)	17
	+	+	3	419 (408–428)	13.[illegible]	.84 (.81 .90)	.34
8	0	+	3	543 (531 556)	7.0	.8 (.80– .83)	14
	+	+	4	502 ± 15	7 8	.89 ± .07	.52
		0	5	16 ± 1	10.2	–.23 ± 11	15
9	+	+	5	27 ± 1	9 3	.84 ± .06	18
10	+	0	5	7 1 ± .06	10.7	1.32 14	17
		+	5	9 5 ± .06	12.[illegible]	75 ± 10	14
11	0	0	3	7 7 (7 1–8.9)	9 3	79 (71 .85)	1
		+	3	7 1 (6.5–8.3)	10.3	.46 (.43 .50)	14
1	0	0	3	10.3 (10.0–10.7)	16.4	52 (51 .52)	09
		+	3	13.2 (10.7 15.0)	17.8	35 (.31 .37)	.06
13	0	0	3	10.2 (8.6–12.9)	10.1	74 (68– 79)	.09
		+	3	1[illegible].6 (12.1 13 6)	1[illegible].6	55 (48– 6)	.04

probenecid, the excretion of urate after administration of probenecid is smaller than the amount derived from glomerular filtration and it must hence be assumed that net reabsorption of urate has occurred under these conditions.

A similar effect of probenecid on the C_u/C_{In} ratio occurs at low plasma levels during infusion of mannitol. In contrast to the results of POULSEN (1955), a small decrease in urate excretion was demonstrated in our study during infusion of physiological saline when reabsorption of urate prevails. The depressing effect of probenecid on urate excretion is presumably due to an inhibition of urate secretion (MOLLER 1965), but the results do not exclude the possibility that probenecid also has an inhibitory action on urate reabsorption. Such an effect might be masked by

the decreased urate secretion under these conditions, but it must be concluded that, if probenecid impedes urate reabsorption, inhibition is far from complete.

On the other hand, there are reasons to believe that the secretion of urate is almost completely stopped after infusion of probenecid. Thus, the C_{Ur}/C_{In} ratios always remain below 1.0 even during vigorous osmotic diuresis. Further the ratio invariably falls to 0.8–0.9 in spite of widely differing values for the control periods. These findings suggest that secretion of urate is virtually eliminated after administration of probenecid. Experimental results in support of this view will be presented in the next paper (MØLLER 1965).

Summary

1. The study reported here confirms an earlier finding (POULSEN 1955) of a depression of the C_{Ur}/C_{Cr} ratio to 0.8–0.9 after administration of probenecid to rabbits made hyperuricaemic by infusion of urate.

2. Probenecid also has an inhibitory action on urate excretion at endogenous plasma levels of urate.

3. Ultrafiltration studies show that urate is not proteinbound.

4. The results support the view that secretion of urate is almost completely inhibited by probenecid.

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Table 2

The effect of probenecid on urate excretion (C_{Ur}/C) t high and low plasma levels of urate.

Experiment no	I fusio of mannitol	Administration of probenecid	No. of periods	Pr (μM l/l)	C_I (ml/min.)	C_{Ur}/C	V/C_{In}
7	0	+	3	3 4	10.8	.84	17
				(317 334)		(78– .87)	
	+	+	3	419	13.2	.84	.34
				(408–428)		(.81 90)	
	0	+	3	543	7 0	.82	14
8				(531 556)		(.80– .83)	
	+	+	4	50? ± 15	7 8	89 ± .02	.52
		0	5	16 ± 1	10.[illegible]	[illegible]23 ± 11	15
9	+	+	5	7 ± 1	9 3	.84 ± .06	18
10	+	0	5	7 1 ± .06	10.7	1.3 _ 14	17
		+	5	9.5 ± .06	1[illegible]2	75 ± 10	14
		0	3	7 7	9.3	79	12
11	0			(7 1–8 9)		(.71 83)	
		+	3	7 1	10.3	46	14
				(6.5–8.3)		(43– 50)	
		0	3	10.3	16.4	.52	.09
12	0			(10.0–10.7)		(.51 .5)	
		+	3	13.2	17.8	.35	.06
				(10.7 15 0)		(.31 .37)	
		0	3	10.2	10.1	74	08
13	0			(8.6–12.9)		(68– 79)	
		+	3	1[illegible]6	1[illegible]6	55	.04
				(1[illegible]1 13.6)		(48 62)	

probenecid, the excretion of urate after administration of probenecid is smaller than the amount derived from glomerular filtration, and it must hence be assumed that net reabsorption of urate has occurred under these conditions.

A similar effect of probenecid on the C_{Ur}/C_I ratio occurs at low plasma levels during infusion of mannitol. In contrast to the results of POULSEN (1955) a small decrease in urate excretion was demonstrated in our study during infusion of physiological saline when reabsorption of urate prevails The depressing effect of probenecid on urate excretion is presumably due to an inhibition of urate secretion (MOLLER 1965), but the results do not exclude the possibility that probenecid also has an inhibitory action on urate reabsorption. Such an effect might be masked by

to allantoin by addition of uricase (Leo) before chromatography only 1 radioactive peak was found, with the R_f value of 0.35–0.40.

These experiments indicate that most of the labelled urate had not been metabolized.

Analytical methods

Urate, inulin and creatinine were determined by the methods previously described (Møller 1965). PAH was measured by the method of Bratton & Marshall (1939) as modified by Smith, Finkelstein, Aliminosa, Crawford & Graber (1945). N was determined by flame photometry. In the experiments with radioactive labelled urate, 50 µl of urine was dissolved in 5 ml of a mixture, consisting of 60 g naphthalene, 4 g PPO 0.2 g POPOP 100 ml methanol, 20 ml ethylene glycol diluted with p-dioxane to final volume of 1 l (Bray 1960), and counted in Packard liquid scintillation counter. No quenching was observed on adding internal standards of urate-8-C[14].

Results

1 Experiments with steady infusion of urate

The stop-flow pattern of a representative experiment with continuous infusion of urate is shown in fig. 1. The $U/P_{Ur} - U/P_{Cr}$ and $U/P_{PAH} - U/P_{Cr}$ values in the first stop-flow samples are of the same magnitude as those in urine collected during the preceeding free-flow periods (shown at the left-hand side of the figure). After the collection of approximately 1 ml of stop-flow urine, the concentration ratios of urate and PAH rise and reach a maximum after collection of approximately 2 ml urine. It can be seen from the figure that the minimal concentration of Na⁺ appears before the urate and PAH peak.

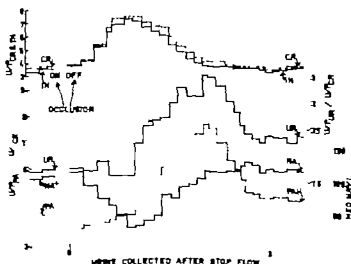


Fig. 1 The stop-flow pattern after continuous infusion of urate (UR), p-aminohippuric acid (PAH), creatinine (CR) and inulin (IN).

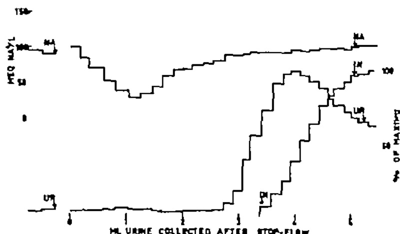


Fig. 2. Compariso between the excretion of urate and I ll I the stop-flow samples after Injection of 100 mg urate and 250 mg inulin 1.0–0.5 min. before the end of the stop-flow period.

A rise in $U/P_U - U/P_{Cr}$ and $U/P_{PAH} - U/P_{Cr}$ ratios was also found in 9 other stop-flow experiments. The shape of the urate curve was similar to that for PAH in all experiments. The concentration of Na^+ was measured in 5 of these experiments, and the minimal concentration of Na^+ was always found to be distal to the urate and PAH peak Further it was noted, in 7 experiments in which inulin was injected late in the stop-flow period that the decline in the urate- and PAH curves occurred simultaneously with the appearance of inulin in the urine.

The experiments thus demonstrate the existence of a proximal secretion of urate, occurring at the same tubular site as secretion of PAH

2 Experiments with injection of urate and inulin late in the stop-flow period

In 4 experiments urate and inulin were injected just before the release of the uretheral occlusion. Probenecid in 2 of these experiments was infused before the stop-flow period.

Fig. 2 shows the results of one of the experiments involving no administration of probenecid The concentration of urate in the distal stop-flow samples is small and corresponds to the level of endogenous urate found in the free-flow periods After collection of approximately 3 ml urine, the urate concentration rises steeply The increase in urate concentration precedes the appearance of inulin in the stop-flow samples. Further a peak for urate concentration is observed before the maximal inulin concentration is reached A similar result was obtained in the second experiment.

The experiments show that some part of the injected urate has perme-



Fig. 3. Comparison between the excretion of urate and inulin in the stop-flow samples after postocclusive injection of urate and inulin. Probenecid at a dosage of 100 mg/kg was infused before the stop-flow period

ated the proximal tubular cells. A much clearer separation between the urate-permeable part of the nephron and the dip in the Na^+-curve is observed in this type of experiment, compared with experiments involving steady infusion of urate (see figs. 1 and 2)

The effect of probenecid on the excretion of injected urate can be seen from fig. 3 Here, the increase in urate concentration coincides with the appearance of inulin in the urine. Besides, the shapes of the urate and inulin curve are almost identical, indicating that urate is predominantly derived from glomerular filtration alone in these circumstances. Probenecid is thus able to inhibit almost completely the secretion of urate.

3 *Experiments with labelled urate*

To determine the permeability characteristics of the various parts of the nephron for urate, 8-C^{14}-urate was injected in 4 experiments at the end of the stop-flow period during infusion of urate In 2 experiments probenecid was infused before occluding the ureteral catheter

Fig. 4 gives the results of one of the experiments after administration of probenecid. The curves in the upper part of the figure show the U/P_{Cr} and U/P_{Cr} values U/P_{Ur} values are a little below the corresponding U/P_{Cr} values in both stop-flow and free flow samples. Hence, the rise in U/P_{Ur} in the distal samples must be due to the abstraction of water during the stop-flow period, and it must be assumed that no net movement of urate has occurred in this part of the nephron during the stop-flow period.

A small amount of radioactivity is found in the stop-flow samples after collection of about 1 ml urine, but the activity rises steeply along with the appearance of inulin in the stop-flow samples.

A similar result was obtained in the other experiment involving probenecid administration. In the 2 experiments involving no administration of probenecid, the most distal part of the nephron was also completely

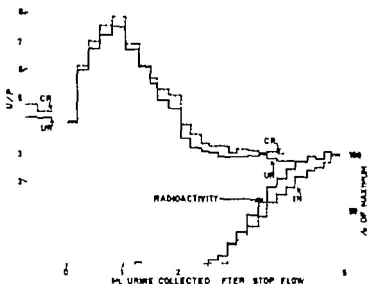


Fig. 4 Experiments with injection of 15 µC 8-C^{14}-urate and inulin 1 0–0.5 min. before the end of the stop-flow period. Urate and creatinine were infused during the whole experiment, and probenecid (100 mg/kg) was administered before occluding the urethral catheter

impermeable to urate, and a zone, characterized by a slight permeability to urate, extended from here to the proximal tubular site of urate secretion.

Discussion

Stop-flow experiments on the excretion of urate in the rabbit have been described in this paper During continuous infusion of urate a rise in $U/P_{Ur} - U/P_{Cr}$ was found corresponding to the proximal part of the nephron. Injection of labelled or non-labelled urate late in the stop-flow period showed that the tubular transport of urate from plasma to tubular fluid took place in the proximal tubule. However after administration of probenecid urate was excreted chiefly by glomerular filtration alone, confirming that probenecid is able to arrest urate secretion almost completely

While this study was in progress, a paper on the renal site of urate transport in the rabbit was published by BEECHWOOD BERNDT & MUDGE (1964) These authors were not able to demonstrate net secretion of urate during infusion of urate under free-flow conditions in most experiments. However in stop-flow experiments a secretory peak for urate corresponding to the proximal tubule was found. Of particular interest was the finding that chlorothiazide lactic acid, creatinine and pyrazinoic acid increased the proximal net secretion of urate. Consistently with the results of our study proximal $U/P_{Ur} - U/P_{In}$ ratios decreased to below 1.0 after

infusion of probenecid. Indeed, these authors succeeded in demonstrating a reabsorptive peak in the stop-flow curve, located at the proximal tubule, after probenecid administration, and this result suggests that both secretion and reabsorption are confined to the proximal tubule. In our study U/P_{Cr} was only slightly below the values for U/P_{Cr} in samples originating from both distal and proximal parts of the nephron. This may be due to the circumstance that in our experiments reabsorption is small relative to the amount of urate secreted during infusion of urate (MØLLER, unpublished results).

As for the most distal part of the nephron, the $U/P_{Cr} - U/P_{Cr}$ ratios in in our experiments were similar to those obtained during free-flow whether probenecid was infused or not. This finding indicates that no net movement of urate occurred here during the stop-flow period. Further the samples originating from this part of the nephron were not radioactive after injection of labelled urate. Urate has thus either moved out of or into the tubular fluid in the most distal part of the nephron which must therefore be assumed to be impermeable to urate. The zone extending from the site of maximal Na reabsorption during the stop-flow period to the proximal tubular site of urate secretion appears to be only slightly permeable to urate.

BEECHWOOD, BERNDT & MUDGE (1964) also found a relative impermeability of the distal part of the nephron to urate in experiments by the interrupted stop-flow technique (MURDAUGH & ROBINSON 1960). Moreover, much higher distal U/P_{Cr} values were obtained during infusion of urea instead of mannitol, indicating that the increased reabsorption of water occurring in these circumstances is not succeeded by reabsorption of urate.

It was noted that the proximal peak during injection of urate in the stop-flow period was confined to a much narrower region than during steady infusion of urate. This is presumably due to the fact that the tubular fluid slowly moves down the nephron during the stop-flow period (OMACHI & MACEY 1959) as a consequence of continued tubular reabsorption of water and dilatation of the tubuli during the stop-flow period. In this way urate, concentrated in the proximal tubule early in the stop-flow period period in experiments involving steady infusion of urate, will be carried down to a more distal site, resulting in a broadening out of the proximal peak.

Summary

1. Urate secretion has been shown by the stop-flow technique to occur in the proximal tubule.

2. Probenecid decreases $U/P_{Cr} - U/P_{Cr}$ ratios to a little below 1.0 in both distal and proximal stop-flow samples in experiments involving steady infusion of urate. Experiments in which urate has been injected late in the stop-flow period show that probenecid is able to inhibit urate secretion almost completely

3 Injection of labelled urate late in the stop-flow period indicates that the most distal part of the nephron is impermeable to urate The intermediary zone, extending from the site of maximal Na^+ reabsorption during the stop-flow period to the proximal tubular site of urate secretion, is slightly permeable to urate.

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The Metabolic Fate of Bis (p-hydroxyphenyl) cyclohexylidenemethane – F 6060 – and its Diacetate – F 6066

By

HANS LARSSON

(Received July 9 1965)

Bis (p-acetoxyphenyl) cyclohexylidenemethane (F6066) (see fig. 1) is a representative member of a large group of asymmetric diphenylethenes, synthetized in the chemical research laboratory of AB Ferrosan (MIQUEL *et al* 1963)

In laboratory animals F6066 is a weak oestrogen with an inhibitory action on gonadotrophin secretion (EINER JENSEN 1965) Clinically the substance has proved useful in the treatment of prostatic cancer (NYLANDER & TERNER 1963 & 1964) The properties in gynaecological practice have been studied by PERSSON (1965a & b) who found that it not only influences the excretion of gonadotrophins in post menopausal women and in those of child bearing age, but also suppresses the excretion of oestradiol-17β. HANNGREN *et al* (1965a & b) studied the distribution of the substance in mice by whole body autoradiography and found a rapid specific accumulation of radioactivity in active corpora lutea. The substance also accumulated in the adrenal cortex, hypophysis and endometrium and in the interstitial tissues of the ovaries and testes. Considerable radioactivity was also demonstrated in the excretory routes, especially in the bile.

The purpose of the investigation described here was to chart the excretory pathways of the compound in different species and to elucidate its metabolism. The study was carried out not only on rats and rabbits, but also on human subjects Their inclusion was justified because long clinical experience has shown that the substance has no toxic side-effects (NYLANDER & TERNER 1964 PERSSON 1965a)

It was soon found that F6066 was readily converted *in vivo* to the corresponding diphenol F6060 (table 1) This substance was therefore also included in some of the experiments described here.

For comparison, use was made of diethylstilbestrol, despite the marked endocrine effects caused by the large doses used of this substance in the metabolism experiments.

The metabolism of diethylstilbestrol has been studied in several species (SIMPSON & WILDER SMITH 1949, TWOMBLY & SCHOENEWALDT 1951, HANAHAN *et al.* 1953, HOPWOOD & GASSNER 1962) and the nature of its conjugates and their excretion may be regarded as established. But in spite of the fact that it has been known for more than 20 years and has been widely used and studied – it has been the subject of two extensive theses – our knowledge of the oxidative catabolism of diethylstilbestrol and other likely metabolic pathways is still only vague (DASKALAKIS 1953, KNOCHE 1963).

Materials and Methods

Compounds used

The compounds used in the metabolic studies and those for comparison of chromatographic and other results are given in table 1.

The ultraviolet spectra were taken in a Zeiss spectrophotometer PMQ II with use of matched 1 cm Ultrasil quartz cuvettes.

Labelled compounds

Fig. 1 shows the synthetic process for marking F6060 and F6066 with ^{14}C at the "methane carbon atom". The marking was done at Ferrosan's chemical research laboratory. ^{14}C-labelled diethylstilbestrol[1]) was obtained from The Radiochemical Centre, Amersham, England. The purity of the labelled substances was examined by paper and thin layer

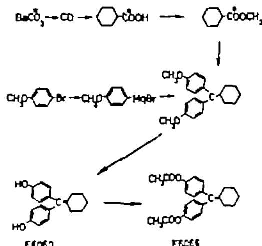


Fig. 1. Reactions in the synthesis of ^{14}C-labelled F6060 and F6066.

1) Monoethyl-^{14}C, specific activity 28.9 μC/mg.

Table 1

Chemical formulas and ultraviolet spectroscopic values for the compounds used.

R_1	R_2	R_3	Number or abbreviation	0.05 N HCl / 50% (v/v) methanol: λ max, mμ	0.05 N HCl / 50% (v/v) methanol: ε·10^4	0.05 N-NaOH in 50% (v/v) methanol: λ max, mμ	0.05 N-NaOH in 50% (v/v) methanol: ε·10^4	Abs. methanol: λ max, mμ	Abs. methanol: ε·10^4
CH_3COO–	CH_3COO–	= (cyclo)C_6H_{10}	F6066	–	–	–	–	247	1.71
HO–	HO–	= (cyclo)C_6H_{10}	F6060	43	1.79	270	2.47	–	–
KSO_3O–	KSO_3O–	= (cyclo)C_6H_{10}	F6108	249	1.50	250	1.58	–	–
GLUCAO–	HO–	= (cyclo)C_6H_{10}	F6096	241	1.70	255	1.77	–	–
CH_3O–	HO–	= (cyclo)C_6H_{10}	F6149	242	1.78	255	2.08	–	–
CH_3O–	CH_3O–	= (cyclo)C_6H_{10}	F6090	–	–	–		42.5	2.10
CH_3COO–	CH_3COO–	< H, (cyclo)C_6H_{11}	F6092	–	–	–	–	227	1.57
HO–	HO–	< H, (cyclo)C_6H_{11}	F6091	234	1.25	250	1.48	–	–
				276	0.27	291	0.42	–	–
HO–		= (cyclo)C_6H_{10}	F6095	246	1.47	257	1.57	–	–
HO–	HO–	= O	6113	255	1.32	240	1.13	–	–
				299	2.13	360	3.03		
HO–	HO–	< H, H	6094	229	1.49	248	1.96	–	–
				278	0.33	295	0.47	–	
Parahydroxybenzoic acid			PHBA	255	1.55	280	1.75	–	–
Diethylstilboestrol			DES	233	1.79	257	2.05		–

chromatography in at least two of the systems given in table .., with subsequent autoradiography and scanning. When the substances showed more than 0.5 of radioactive impurities, they were re-crystallised from suitable solvents to at least 99.5 % radiochemical purity as judged from scanning and evaluation of the chromatograms in combination with ultraviolet spectra.

Experimental procedures

A. *Animal experiments* The substances were as a rule dissolved in oliv oil and given either by mouth or subcutaneously The volume given to rats was 0.2 ml and to rabbits 4 ml per animal. Rats[1]) were kept in metabolism cages, three animals to the cage. The diet consisted of water and raw beef *ad libitum*. The excreta were collected at intervals for periods up t 4 days. After the experiment the animals were killed by bleeding, the blood was collected, and some internal organs were dissected and isolated. Those specimens and extracts not studied immediately were kept at −25

In the experiments rabbits[2]) a similar procedure was used, but each a imal was kept in separate metabolism cage. The animals were fed on carrots and water *ad libitum*.

In a few experiments in which the distribution of the radioactivity in the rat and rabbit was determined, the imals were given one dose t the beginning of the experiment and a second equal one 4 days later After further 5 hours they were killed, the blood was collected, and the organs were removed.

B. *Trials on h ma subjects* ^{14}C F6066 was dispensed in gelatin-capsules containing 300 mg live oil and taken by the subjects together with 1 or 2 uncoated tablets of non-labelled F6066 à 200 mg.

Urine was collected in 4-hour volumes for period up to 6 days. I one study in which F6066 tablets were given preoperatively to three patients with *gallstone the g llbladder* content was set aside at operation and afterwards treated largely by the extraction method described under B below

Extraction of biological material

A. *Urine* The procedure used for the extraction of metabolites of F6066 and diethylstilbestrol from the urine was briefly as described below The sample w acidified with 5 M H_3PO to pH $\leq$ 2 and then extracted with ether (A). By distribution bet een ether and 0 5 M $NaHCO_3$ and 0.2 N N OH, the extract A was divided i to conjugate fraction (B) and a "free phenol fraction (C), respectively each of which was then separated hromatographically Hydrolysis was sometimes done with β-glucuronidase before extracti g the substances from the urine, but the best result ere obtained when the extracted conj gate was subjected t enzymatic hydrolysis, f r th rt as possible to identify the conjugated product chromat gr phically and determine their quantities.

B. *Faeces nd organs* The samples, either fresh or freeze-dried, were ground in mortar with w shed quartz sa d The biological material w extracted three times by grinding the samples with a suitable olume of bsolut ethanol, sually 10 ml/g fresh organ. This method proved mor effecti than the So hlet-extraction f minced samples nd had the advantage that the extract not exposed t heat The ethanol was filtered nd the crude extract was mixed with equal olume of w ter The mi ture wa then h ken thoroughly with twice tl olume of cyclohexane After centrif gation the tw phases ere separ t d as completely possibl nd were hen worked p separately In the cyclohexane phase which contained lipophilic subst nces, only mall amo nt of F6066 deri tives ere

1) White rats, Ferrosan inbred str (Bodyweight 180–210 g)
2) White rabbits, tra n of Statens Seruminstitut, Copenhagen.

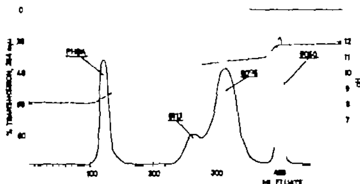


Fig. 2. Separation of 0.?? mg PHBA, 0.40 mg 6113 2.42 mg 6096 and 0.59 mg F6060 on a 40 cm Sephadex column. Elution performed with gradient 0.1 M $NaHCO_3 \rightarrow 0.1$ N-NaOH.

——— % transmission at 254 mμ

——— pH

demonstrable in the subsequent experiments. Unchanged F6066 would undoubtedly have appeared almost quantitatively in this lipophilic phase (distribution constant K for F6066 in the system cyclohexane/50 % (v/v) ethanol > 100). The ethanol-water phase, on the other hand, contained the more polar compounds, including F6060, which have been shown to be degradation products of F6066. Fo separatio and purification of the polar metabolites the same method was used as for the urine, though not until after the bulk of the ethanol had been removed by evaporation of the extract to one third of its original volume. After acidification with 5 M H_3PO_4 to pH ≤2 the metabolites were extracted with ether by the same method as for urine.

Column chromatography

It proved necessary to devise column chromatographic system that not only separated phenolic substances from their conjugates, but also purified them from all the polar compounds normally occurring, for example, in urinary extracts. As filling for the column we used Sephadex ® G-25, which has been used by NILSSON (1962) for similar separations in the isolation of the excretory products of isoflavones and by BELING (1963) for purifying and determining conjugated oestrogens in the urine of pregnant women. The apparatus and the method used for column chromatography of excretory products have been described previously (LARSSON 1964). In principle the method is briefly that a gradient 0.1 M-$NaHCO_3 \rightarrow 0.1$ N-NaOH is used as the mobile phase, which means that relatively nonpolar compounds can also be eluted without the use of large volumes of eluant. The supply and interruption of the gradient, like the elution and washing, is performed automatically. The pH of the eluate and its transmission at 254 mμ is recorded continuously during separation with the aid of the bsorption curve fractions of interest can be selected for further study. A model experiment with some of the substances given in table 1 is illustrated in fig. 2.

Paper and thin layer chromatography

A series of chromatographic systems for paper and thin layer chromatography were used for testing the purity of the substances and for the fractionation of biological extracts.

A. *Material* Paraffin-impregnated paper was prepared in the way previously described (LARSSON 1963).

Acetylated chromatographic paper (MN 214 Ac) was purchased from Macherey N gel & Co, Duren Germany Two degrees of acetylation 10 and 20 % acetyl, were used.

Whatman 1 paper was impregnated with formamide[1]) for the systems M and M4 by dipping it in a 30 % (/v) solution of formamide in methanol and then allowing it to dry in the air for 10 minutes, after which the test solutions were applied.

Thin layer plates, 5 × 20 and 20 × 20 cm, were prepared with Silica gel HF 254 + 366 (Merck) with a Desaga applicator layer thickness 300 μ. After the plates had dried in the air they were activated by heating for 30 minutes at 100° and then stored in a desiccator until used.

B. *Method* As rule, the substances and extracts were dissolved in bsolute ethanol and applied to the chromatograms as spots, or for contaminated extracts preferably as a row of spots forming a 15–20 mm long band.

After development and air drying, the chromatograms were studied in ultraviolet light (Hanovia Chromatolite, 254 mμ) and the absorbing spots were outlined. All the substances except 6091 and 6092 could be demonstrated in amounts of 1 2 μg/cm² On spraying with Folin-Ciocalteaus reagent and subsequent treatment in an ammoniacal atmosphere (MITCHELL & DAVIES 1954) ll the compounds with free phenol groups gave a blue colour Presence of the esterified compounds could also be demonstrated with the same reagent, though not until after hydrolysis *in situ* with alkali.

Chromatographic results for the substances studied are given in table 2.

Elution of the substances from thin layer chromatograms was performed with a modification of the pparatus described by RITTER & MEYER (1962), in which the extraction thimble was replaced by small glass filter plate.

Measurement of radioactivity

The total activity in the urine and extracts was determined by evaporation of suitable volumes on 1½ or 2 inch aluminium planchets and measurement in a Nuclear Chicago Low Background" automatic planchet counter The self-absorption of the samples was determined with the aid of internal standards, and the values obtained were corrected accordingly

The distribution of radioactivity in the paper and thin layer chromatograms was determined with a Vanguard Automatic Chromatogram Scanner provided with ADS (utomatic data system).

Kodirex ® film w used for production of the utoradiograms.

Results

Excretion in rats

Only ^{14}C labelled substances were used in the various experiments on rats. The purpose was, first, to determine the excretory pathways and the rate of excretion of the substances and, secondly to obtain information about their metabolism and distribution. The results obtained in two comparative experiments when ^{14}C F6060 and ^{14}C F6066 were given

[1]) Merck reagent grade

Table 2

Paper and thin layer chromatographic results f r the compounds named in table 1

Number or abbreviation	C4	C8	D6	E4	H1	H5	M2	M4	P1	P3	P8	P10
	R_F 100 in solvent system											
P8066	79	01	25	77	~95	94	93	91	77	84	18	60
P8040	87	47	34	40	9	[illegible]	40	34	47	37	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16063	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
6113	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
6094	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
P11BA	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 2 continued

		Sol ents	Proportions	R	g t me	Technique¹)
Whatman 1 paraffi impregnated	C4	methanol/w t r	3/1		5h	a
	C8	methanol/water	1/2		5h	a
Macherey and N gel, MN 14 10 acetylated	D6	methanol/wate	1.5/1		3h	a
70 acetylated	E4	methanol/water	3/1		3h	
Whatman	I11	n-butanol, 2N-ammonia	1/1		16h	d
	I15	toluene/tert. butanol/acetic acid/water	4/1/1 5/3.5		7h	d
Whatma 1 formamide impregn ted	M	chloroform			2h	d
	M4	toluene			3h	d
Silica g 1 HF 254 + 366 300 μ	P1	chloroform/acet ne	80/70			a
	P3	benzene/methanol	60/10			a
	P8	cyclohexane/di-iso-butylketone/methanol	80/20/2			a
	P10	cyclohexane/di-isobutyl-ketone/methanol	80/70/10			a

References Syst ms I11 and I15 Sc neide & Lewbart (1959) M and M4 Zaff n et (1953).

¹) = scending, d = descending.

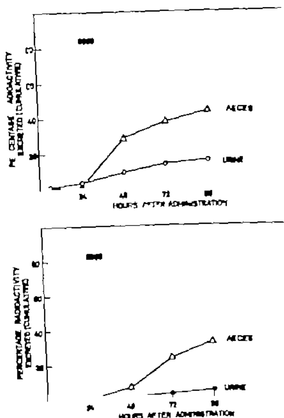


Fig. 3. Excretion of radioactivity after single subcutaneous doses of ^{14}C-labelled compounds to rats.
a. F6060 b. F6066
Each graph represents pooled excreta from 3 animals.

by mouth and subcutaneously are shown in fig. 3 [illegible] is clear that the bulk of the two substances, irrespec[illegible] administration, was excreted in the faeces. The ra[illegible] particularly when the substances were given sub[illegible] mortem examination 96 hours after administering [illegible] of oil were found at the site of injection. Extraction [illegible] the radioactivity showed that 5.1 % of ^{14}C-F66[illegible] depot at the site of injection. The corresponding [illegible] compound ^{14}C F6060 was 0.9 %.

The purpose of the next series of expe[illegible] excretion and deposition of orally administer[illegible] all ^{14}C-labelled. The results are given in fig[illegible] similarity between the two diphenols F6060 [illegible]

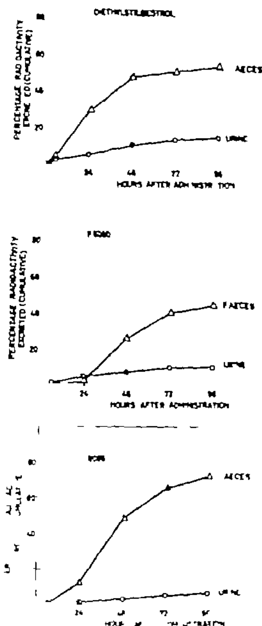


Fig. 4 Excretion of radioact dy fter si gle oral doses f 14C-labelled compounds to rats.
Diethylstilbestrol b. F6060 c. F6066
Each graph represents pooled excreta from 3( ) r 6(b) animals.

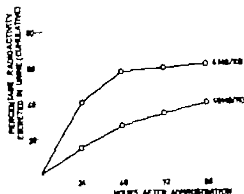


Fig. 5 Excretion by rabbits of radioactivity in urine after single oral doses of ¹⁴C F6066.

a slightly different picture, with excretion of a smaller proportion in the urine than of the other two substances, but a larger proportion in the faeces.

Excretion in rabbits

Similar experiments with ^{14}C F6066 were also carried out on rabbits. Two rabbits were given 4 and 40 mg of ^{14}C F6066 per kg bodyweight.

Strangely enough, it was found that the rabbit, unlike the rat, excreted the bulk of the radioactive substances in the urine. The faecal excretion did not exceed 3.5 / of the dose given. That diethylstilbestrol in rabbits is also excreted mainly in the urine has been shown by SIMPSON & WILDER SMITH (1949)

The cumulative excretion of radioactivity in the urine is shown in fig. 5 The smaller dose showed a higher rate of excretion. Other experiments with nonradioactive substances showed the same tendency

Excretion in human beings

Fig. 6 shows the cumulative radioactivity of urine excreted after administering 1-2 200 mg tablets of F6066 at the same time as an amount of ^{14}C-F6066, corresponding to 6-11 10^6 cpm The results show that the renal excretion of the radioactive substance is higher than in the rat, but lower than in the rabbit. A considerable proportion of F6066 or its metabolites is thus probably excreted in the faeces. No determinations could be made of the activity in the faeces satisfactory accuracy could only have been achieved with much larger doses of ^{14}C, but this was impossible on ethical grounds.

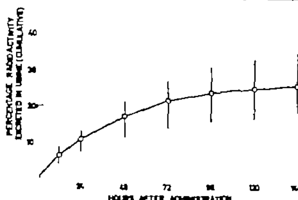


Fig. 6. Average urinary excretion of radioactivity by three women after single doses of 14C-F6066.
Verticals indicate the ranges.

In one experiment with the nonradioactive substance, 400 mg of F6066 was given by mouth one day before operation to three patients with gallstones. In all three bladder-bile samples collected at operation were found to contain F6060-monoglucosiduronic acid (substance 6096). One of the samples contained $\leq$0.2 mg 6060/ml in conjugated form, the other two much more, namely 2 mg and 3 3 mg 6060/ml. These figures clearly do not justify drawing any far-reaching conclusions, but they do show that the bile must be taken into account when investigating the excretion of F6066

The nature of the excreted products

The urines of the various test subjects were analysed for excretory products by the method described under "Materials and Methods" and three extracts were obtained A B and C These extracts were chromatographed on a 40 cm Sephadex column and in some of the systems given in table 2. The proportion of radioactive products extracted already suggested how far the substances had been metabolised to polar products. No differences in this respect were found between F6060 F6066 and DES Fig. 7a shows an example of the distribution of the radioactivity in extracts of the urine and faeces from rats after oral administration of 14C F6066 Chromatography in solvent system P1 of the radioactive extracts from faeces showed that the bulk of the activity was present in a compound with $R_F = 0\ 45$ (Metabolite III in fig. 8a) Small amounts of other products were also demonstrated. The picture yielded by the urine (fig. 8b) was entirely different. Of metabolite III only 1 1–1 4 / of the

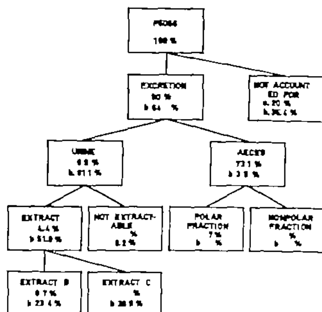


Fig. 7 Excretio of radioactivity within 96 hours after single dose of ^{14}C F6066 to
a. rat (8–12 mg/kg body weight)
b. rabbit (4 mg/kg body weight)
Also shown is the distribution of radioactivity among the various extracts of urine and faeces. All percentages are in terms of dose = 100 %.

dose was recovered, whereas all the other products except the minor metabolite IV were more polar Corresponding results were obtained by chromatography on a Sephadex column (fig. 9) extraction of the radioactive fractions obtained and thin layer chromatography of these extracts. The eluate was found to contain the metabolites listed in fig. 8.

In excreta from rabbits, of which, as mentioned earlier the urine contained the bulk of radioactivity emanating from ^{14}C F6066 the proportion of the "conjugate extract B was much larger than in those from rats, especially after the large dose (40 mg/kg) In fig. 7b the distribution of radioactive products in faeces and urine from rabbits is shown. Fig. 10a shows the distribution of the radioactivity in the eluate obtained on Sephadex chromatography of extract A from rabbit urine. Further separation into extracts B and C yielded relatively pure solutions, from which metabolites were isolated by Sephadex chromatography (Fig. 10b & c)

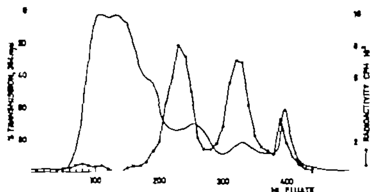


Fig. 11 Separatio of portion of pooled human urine extracts A.
Column Sephadex G-25, medium.
Eluent 0 1 M $NaHCO_3 \rightarrow$ 0.1 N-N OH.
Radioactivity has been determined by plating and counting 0.2 ml portions of the individual 10 ml fractions.

From human urines more than 90 / of the radioactive products could be ether extracted (extract A) On Sephadex chromatography of this extract only a small part of the radioactivity was found in the fractions corresponding to F6060 (390–410 ml) Most of the substance given had been converted into polar excretion products. (Fractions 180–270 and 280–380 ml in fig. 11)

Isolation and identification of metabolites

In an attempt to isolate the products excreted in the urine, 2 rabbits were given daily oral doses of 400 and 2000 mg of F6060 for 3 days. The urine was collected for 6 days, and the products were extracted by the method described After evaporation of the solvent of "extract B 2 g of a syrup-like substance was obtained. After re-crystallisation 3 times from acetone-benzene, 65 mg of a slightly yellow substance was obtained this on chromatography in the systems H1 and H5 gave only one ultraviolet absorbing spot, which also showed a positive reaction with Folin-Ciocalteau s reagent.

Of the substance 0 5 mg was titrated with 0 02 N–NaOH in an automatic titrator (Radiometer TTT1) The equivalent weight was calculated from the titration curve and found to be 451 and the apparent pK_a 3 1

Of the compound 0 202 mg was incubated for 36 hours at 37° with β-glucuronidase, after which the hydrolytic product was extracted from the solution with ether After evaporating and dissolving the residue the ultraviolet spectra were determined in 0 05 M–NaOH and HCl, in 50 / (v/v) methanol. Except for somewhat higher extinctions at wavelengths

<225 mμ, the spectra were the same in acid and alkaline solutions as those of F6060. With the aid of the extinction at 270 mμ in alkaline solution the amount was calculated to be 0 117 mg F6060 That the product of hydrolysis was indeed F6060 was also verified by chromatography in three systems.

The substance isolated from the "B-extract" therefore most probably consisted of F6060-monoglucosiduronic acid and was designated F6096.

	Calculated	Found
M.w	= 456 4	451
/ⁱ6060	= 61 4	58.0

The substance is readily soluble in alcohol, acetone and alkali soluble in water and almost insoluble in benzene. For ultraviolet spectrum, see table 1

The conjugate proved relatively unstable when kept in 1 N HCl for a day crystals of F6060 formed.

Extract C from the above-mentioned rabbit urine was diluted with 1 N-HCl, and about 200 mg of a colourless crystalline substance was isolated. After recrystallization twice from 70 / (v/v) ethanol the substance showed the same ultraviolet spectrum as F6060 On paper and thin layer chromatography in 3 of the systems given in table 2 the compound gave the same R_F-values as the reference substance F6060

Because of the low water solubility of F6060 it is probable that fresh urine contained only small amounts of this compound, but owing to hydrolysis and enzymatic processes in the urine, and to the acid milieu during extraction, it was released from the original conjugate excreted. The metabolites of F6060 and F6066 found to occur in urine extracts were numbered I–VI in order of decreasing polarity in the chromatographic system P1 (see fig. 8.)

Metabolite IV (fig. 12) occurred in only minute amounts in extracts from the urine and faeces of rats. The R_F-value of the substance in system P1 was 0.55–0 60, i.e. a value between those for F6060 (R_F = 0.47) and F6066 (0 77) Since it could be demonstrated only as an excretory product of F6066 but not of F6060, it was assumed to represent a stage in the deacetylation F6066→F6060, probably the "monoester"

Because of the low concentration in the extracts and failure to synthetize the corresponding compound, attempts to identify further the substance by means of its spectra were abandoned.

Metabolite III occurred in all extracts from the faeces and from the urine since it satisfied the five criteria listed below it was considered identical with F6060.

Table 3

Excret[o]ry products of ^{14}C F6066 w[i]thin 96 hours (ma[x] 120 hours) after si[n]gle doses.
The values are based on determinations of the radioactive metabolites on thin layer plates and in eluted fractions from the Sephadex column. As [a] rule, [a]t least three determinations were mad[e]; the figures represent radioactivity as percentages [o]f the doses. Ranges are given in brackets.

Metabolite	Rat				Rabbit		M[a]n
	Oral		Subcuta[ne]ous		Oral		Oral
	Urine	Faeces	Urine	F[a]eces	Urine	Faeces	Urine
Ia	0.7 (0.4–0.8)		1.5	–	10.2 (8.8–1[illegible].0)	–	11.9 (8.6–13.4)
Ib					13.2 (13 0–13.5)		1 1 (0.7 1 5)
II	2.3 (2.0–2.7)	6.5 (4.2–7.9)	1 8	5 4 (4.6–6.1)	8.0 (5.5–10 0)	0.3 (0.[illegible]–0.5)	12.3 (0.0–17.0)
III	1 3 (1 1–1 4)	58.8 (52.1–60.7)	0.9	30.3 (26 0–34.2)	20.5 (15.0–25 7)	3 1 (2.9–3.3)	1 3 (0.9–[illegible].0)
IV	0.1 (0.05–0.2)	1 7 (0.2–2.8)	<1 7	1.5 (1 4–1 6)	~0.2	~0.1	0.5 (0.4–0.7)
F6066	–	6.0			–	<0.05	–

Table 4

Distribution of radioactivity in rats and rabbits 5 hours after oral dosing of ^{14}C-labelled F6060, F6066 and diethylstilbestrol.

Dose	Radioactivity cpm 10^3/g wet weight								
	Blood	Ovaries	Testes	Uterus	Adrenals	Kidneys	Liver	Bile	Leg muscle
6,84 10^6 cpm diethylstilbestrol to three rats	0.24	3.92	–	3.93	0.40	0 97	6.02	–	0.23
3,22 10^6 cpm F6060 to three rats	0.13	9 60	–	4 53	0.[illegible]3	[illegible]50	2.14		0.10
6,50 10^6 cpm F6066 to three rats	0.12	[illegible]14		4 18	0.66	0 72	2.40		0.13
3,5 10^6 cpm F6066 to two rabbits	0.009		0.047	–	0.33	0.17	0.17	6.85	0.011

appears to be a reasonable explanation for the relatively slow excretion of the degradation products of F6066 in the urine.

The experiments showed that F6060 and F6066 in the rat have a metabolic fate similar to that of diethylstilbestrol One of the most striking findings in this investigation has been the large difference between the metabolic fates of F6066 in different species, both in the excretion routes and in the nature of the products excreted.

Summary

The excretion and metabolism of bis(p-acetoxyphenyl) cyclohexylidene methane (F6066) and the corresponding non-acetylated compound, F6060, were studied in rats, rabbits and human subjects. Use was made of diethylstilbestrol as a reference substance in the metabolic studies metabolically it proved to resemble F6060 The most reliable results were obtained with ^{14}C-labelled substances.

Measurement of the radioactivity in the urine and faeces from rats given ^{14}C F6060 and ^{14}C F6066 showed that the bulk of the substances is excreted in the faeces, whether the dose is given subcutaneously or by

mouth, whereas in rabbits the bulk of the radioactivity is excreted in the urine

Human urine collected for up to 6 days was found to contain 17–35 / of the dose ^{14}C F6066 given and that mainly in metabolised form.

The rate of excretion in all the species studied was relatively low several / of a single dose appeared in the excreta later than on day 4

The urinary excretion products were found to consist not only of metabolised and conjugated substances, but also of highly polar compounds not extractable with ether from an acid solution

In the faeces of rats and rabbits the main product was F6060 even on administration of F6066, but here, too a small proportion of the substance had undergone metabolic conversion to polar products.

In bile from the rabbit and from human subjects F6060-monoglucosiduronic acid was identified This compound was isolated in pure form from rabbit urine also

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Interfering Substances by Determination of Poisons in Autopsy Material. V 5-Hydroxymethylfurfurol

By

Bent Kæmpe

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During extraction of poisons in autopsy material there may be produced as previously stated (Kæmpe 1965), substances that interfere with the spectrophotometric analysis of the extract. In the study reported here one of these substances, 5-hydroxymethylfurfurol, has been further studied. The absorption curve for this substance in the ultraviolet region has often been seen on analysis of fresh autopsy material and foodstuffs (e.g. potatoes and bread) for poisons of an acid character when using the so-called Stas-Otto extraction method of isolation. Isolation and identification of the substance will be described below and a method will be given for separating this from salicylic acid and barbituric acids, which appear in the same extraction phase.

Technique

General technique

Physical constants are determined as described previously (Kæmpe 1964). The substance is placed between two plates of sodium chloride for infrared spectrophotometry.

As object of analysis we used a single human liver stored whole for 2½ months at 4–5 (The death of the person concerned had probably been caused by a blow on the carotid).

Extraction (a modification of the Stas-Otto method).

To 300 g minced liver tissue were added 1 litre of 96% ethanol and crystalline tartaric acid to give an acid reaction. The mixture was heated on a boiling water-bath for 1–2 hours. The alcoholic layer was centrifuged off and the residue extracted twice, each time with 300 ml of 96% ethanol. The combined alcohol extracts were evaporated to 100–200 ml on a steam-bath at a pressure of 70 mm Hg. To the resultant extract 500 ml of 99% ethanol were added the mixture was again centrifuged and then evaporated to about 100 ml. The resultant concentrate was cooled down and filtered, after which the alcohol was distilled off. Evaporation to dryness was avoided by gradual addition of water and

few millilitres of 2 N-H_2SO_4. The cooled filtered aqueous solution (about 50 ml) was extracted three times, each time with a large volume of ether. Anhydrous sodium sulphate was added to the combined extracts. After filtration and then evaporation on a steam-bath to 5 ml, the last remnant of ether was removed by standing at 25°. The residue was heated three times on a boiling water-bath, each time with 20 ml of water. A few millilitres of 1 M phosphate buffer pH 6.6, were added to the combined, cooled and filtered aqueous solutions. This was extracted five times, each with an equal volume of chloroform. The combined dried chloroform extracts were evaporated to about 50 ml. The chloroform extract was shaken once with 10 ml of 0.02 N borate buffer pH 10.8, which was extracted with 50 ml of chloroform before being discarded. The two chloroform extracts were mixed, evaporated to 1 ml and then transferred to ½ mm thick activated silica gel-G plate. The plate chromatogram was developed with a mixture of equal parts of chloroform and ether (NIELSEN, unpublished method 1965). The material in the area round R_F = 0.4 was scraped off and eluted twice each time with 15 ml of methanol. The methanol extracts were centrifuged, after which the clear supernatants were evaporated on a boiling water-bath at a pressure of 70 mm Hg. The residue was transferred with chloroform to Eder's sublimation tubes. A clear fluid (1–2 mg) was collected by sublimation at 70° and a pressure of 5 mm Hg.

Results

The spectra of the amorphous substance isolated from liver have been recorded in the infrared and the ultraviolet regions (see figs. 1 and 2, respectively). A dark spot with R_F value = 0.74 is seen in 254 mμ light on Whatman paper no. 1 developed with a mixture of n-butanol and water in the ratio of 100: 15 (WALKENSTEIN *et al.* 1958).

The constants found corresponded fairly closely to the data in the literature for 5-hydroxymethylfurfurol (5-H.M.F.). A sample of pure 5-hydroxymethylfurfurol, Fluka, was used for comparison. Its spectra in the infrared and ultraviolet regions have been recorded (see fig. 1). Its R_F values, with the plate and paper-chromatographic systems described above, were 0.42 and 0.74 respectively (like meprobamate). The substance was localised *on the plate-chromatogram as a yellow spot by* spraying with the chlor-benzidine reagent described by VIDIC (1959).

Comparison of the results for the amorphous substance isolated from liver with those for 5-hydroxymethylfurfurol showed these materials to be identical.

As 5-hydroxymethylfurfurol shows an intense absorption band at about 280 mμ (5 μg 5-H.M.F./ml ~ E = about 0.8 with a light-path-way of 1 cm) and a weaker band at about 225 mμ (see fig. 2), its presence will interfere with the spectrophotometric determination of substances that are co-extracted with ether from an acid aqueous phase, e.g. salicylic acid and 5,5-substituted barbituric acids. Attempts have therefore been made to separate 5-hydroxymethylfurfurol from the substances mentioned.

650 700 800 900 1000 1100 1200 1300 1400 1500 1600 cm⁻¹

Fig. 1. *Infrared spectra of the substance isolated (upper curve) and 5-hydroxymethylfurfurol (lower curve) in NaCl.*

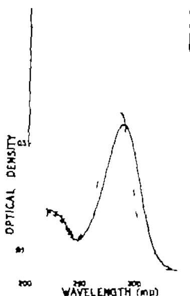


Fig. 2. *Ultraviolet spectra of the substance isolated (5-hydroxymethylfurfurol) in 0.02 N borate buffer pH 10 ...... in 0.1 N HCl – – – and in 0.1 N NaOH ——*

Separation of 5-hydroxymethylfurfurol from salicylic acid and 5,5-substituted barbituric acids

On Whatman paper no 1 developed with ALGERI & WALKER's (1952) mixture, used in this Department for determinations of barbituric acids, 5-hydroxymethylfurfurol has an R_F value of 0 78 Accordingly this system is unsuitable for separating this substance from the ordinarily occurring barbituric acids (R_F 0.5 – 0 8)

Extraction experiments

Barbituric acids and salicylic acid are separable from co-extracted 5-hydroxymethylfurfurol by repeated extractions of the 5-hydroxymethylfurfurol with an abundant volume of ether or chloroform from an alkaline aqueous phase (pH > 11) Barbituric acids and salicylic acid are well-known to remain in the alkaline aqueous phase.

Table 1 shows that pentymal (same R_F value as 5-hydroxymethyl furfurol on ALGERI & WALKER's (1952) system) can be separated from 5-hydroxymethylfurfurol by a single extraction with chloroform (or ether) from an aqueous solution at pH 5 0, to which has been added $Na_2S_2O_5$ Unlike pentymal, 5-hydroxymethylfurfurol will remain in the aqueous phase.

Spectrophotometry

Absorption maxima for 5-hydroxymethylfurfurol at about 280 mμ, measured at pH 2 and pH 10 but not at pH 13 disappeared on adding

Table 1

Partition coefficients at room temperature for 5-hydroxymethylfurfurol and pentymal.

Substance	Aqueous phase against	Chloroform	Ether
5 hydroxymethylfurfurol	0.1 N-H_2SO_4	3 3	3.0
	Borate buffer pH 10.5	2.7	2.9
	0.1 N-NaOH	3 1	3 4
	Phosphate buffer pH 5 with 8 mg $Na_2S_2O_5$ per millilitre added	almost nil	
Pentymal	Phosphate buffer pH 5 with 8 mg $Na_2S_2O_5$ per millilitre added	almost all	

$Na_2S_2O_5$ to the solution. On spectrophotometric determination of such substances as salicylic acid, for instance, which absorb at about 280 mμ, interference of 5-hydroxymethylfurfurol can therefore be eliminated by adding $Na_2S_2O_5$ to the measuring solution as well as to the reference cuvette.

Discussion

During heating of hexoses, especially ketohexoses, in the presence of diluted acid 5-hydroxymethylfurfurol is produced

As liver tissue normally contains glycogen (glucose) and also glucose and fructose after intake of sugar the substance must be expected to be present in liver extracts obtained by such extraction as that described above under "General technique"

The characteristic absorption curve in the ultraviolet region (see fig. 2) must likewise be expected during spectrophotometric analysis for poisons in gastric contents including carbohydrate-containing foodstuffs (e g. potatoes and bread) when extraction is performed as described above.

Summary

One of the impurities often seen on spectrophotometric analysis for poisons of even fresh post mortem livers and gastric contents extracted by the so-called Stas-Otto method has been identified as 5-hydroxy methylfurfurol Methods have been described for separating it from barbituric acids and salicylic acid, as well as for eliminating its inter ference during spectrophotometric analysis for poisons.

Acknowledgement

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From the Pharmacological Department AB Ferrosan, Malmö, Sweden

Bis (p-acetoxyphenyl) cyclohexylidenemethane (F 6066), A Non-Steroidal Compound with Pronounced Effects on the Reproductive System

By

Niels Elner-Jensen

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Since the discovery of the oestrogenic properties of stilboestrol (Dodds, Goldberg Lawson & Robinson 1938), the search for new non-steroidal oestrogens has mainly been concentrated on derivatives of stilbene and dihydrostilbene, but other substances have also been studied, e.g. derivatives of diphenylmethanes, diphenylpropanes and triphenylethylenes.

In an extensive study of triphenylethylene derivatives, initiated by Mentzer & Dat Xuong 1946 and later continued by Miquel, Buu-Hoi & Royer 1955 and Miquel 1958 alkyl groups instead of a benzene ring were introduced at the β-carbon atom. Later on, the alkyl groups were replaced by a saturated cyclic group (Miquel, Wåhlstam, Olsson & Sundbeck 1963)

One of those substances, bis(p-acetoxyphenyl)cyclohexylidenemethane[1]) F6066 (see fig. 1), was selected for clinical trials after having shown a relatively stronger antigonadotrophic than oestrogenic effect in animal experiments (table 2, and Barany personal communication 1962) In

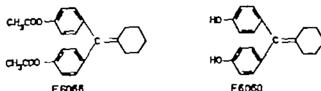


Fig. 1 Formulas of bis(p-acetoxyphenyl)cyclohexylidenemethane (F6066) and the deacetylated compound (F6060).

[1]) The substance was synthetized by Ferrosan Ltd, Malmö, Sweden.

The method of SPENGLER (1958) was modified, with injections o two consecutive days and observation f r 5 days. During the first and second days the animals were injected one hour before the eating period, and the food consumptio was measured after and 5 hours u til and including the fifth day Each dose level was given to 10 animals. In this experiment the values f day 1 (a Monday) are recorded, with the reservatio that no training of the animals could be carried out o Saturdays and Sundays. The results are given for 2 hours.

Reproduction

10 male and 10 female rats weighi g about 100 g were i jected subcutaneously five times week f 6 weeks with 2 mg per animal of a soluti n of F6060 i olive oil. During this period 5 animals of the same sex were caged together but fter the 6th week 1 male and 2 females were placed in the same cage.

To distinguish changes i fertility in each sex after dosing, some of the animals were caged together with animals of the opposite sex treated with live oil for the same period. The different combinations are presented i fig. 6, from which it can also be seen that some of the animals were given injections for further 3 weeks.

Exactly the same experiment was carried out with F6066 (fig. 7).

Anabolic ffect on male nd castrated mal rats

In co nection with the previously described test of the effect on testes and prostate, the levator ni muscles were taken out and weighed. In castrated animals and increase in muscle weight was considered to indicate n anabolic effect. (HERSHBERGER, SHIPLEY & MEYER 1953).

Lactation

I order to study the effect on lactation, F6066 was given a dose of 5 mg per animal subcutaneously to lactating rats o days 4–9 after the birth of the young. Each group consisted of 4 n mals, each with 6 young. A group in which each animal received 0.1 mg oestradiolbenzoate + 1 mg progesterone subcutaneously served as control.

Diuretic effect

F6066 was given orally dissolved in olive oil, t groups of 4 rats t a dosage of 30 mg/day by stomach tube for 2 days Immediately after the 2nd administration, each rat was placed over funnel d the urine was collected for 72 hours. The male ats weighed on average 180 g and the female rats 170 g The results are shown in table 7 which gives the relative values of the excretio th values of the corresponding control group being taken as 1 0. Sodium and potassium were determined by flame photometry chloride by complexometric titration. Creatinine was determined col rimetrically (LARSEN & NIELSEN 1963).

Antiinflammatory effect

The methods described by MEYER, STUCKI & AUFDERBROOK 1953 and by CYGIELMAN & ROBSON 1963 were slightly modified. Male ats weighing bout 150 g were adrenalectomized under ether anaesthesia. During th test period the animals were given 0 9 % saline and ordinary water d libitum to drink. Two non-sterile dental pellets (No 4 Richmond Dental Cotton Co) were implanted subcutaneously iat two spots the ventro-lateral wall f the bd men. This was done simultaneously with the drenalectomy but through two new incisions in the skin The animals were given injections subcutaneously nce day as far

Table 1

Acute oral toxicity of F6066 and stilboestrol in mice.

Weight of animal gram	Susp. gum arabic dissolved in olive oil	Administration daily number of days	LD50 mg/kg/day
19–24	F6066, susp	1	>12500[1])
9–11	F6066, solution	2	>1600[1])
22–25	F6066, solution	2	>1600[1])
19–25	Stilboestrol, solution	2	566 (408–784)

[1]) The highest dose tested. 6 animals per dose were used.

from the pellets as possible. The first day was the operation day. On the 8th day the animals were killed and the pellets, together with the firm tissue around them, were weighed wet and after storage at 100° for a minimum of 12 hours. Five animals were used per dose, and the substance was dissolved in 0.2 ml olive oil. The prednisolone acetate (Meticortelone, Schering Corp.) was given subcutaneously as a suspension.

Antigestagenic effect

A modified CLAUBERG' test (1930) was carried out on immature female rabbits (600–800 grams). After priming with 0.5 μg oestradiolbenzoate for 7 days, combinations of 0.2 mg/kg progesterone[1]) and various doses of F6066 were injected subcutaneously for another 7 days. On the 15th day the animals were killed, and the uteri were examined histologically.

Results

Acute toxicity

No acute lethality of F6066 was observed. At low doses the animals showed no objective signs. At higher doses they showed diarrhoea, increased motor activity, tactile hypersensitivity and anorexia for 24 hours. Similary treated control animals given olive oil by stomach tube showed anorexia within 4–6 hours and sometimes diarrhoea (table 1).

Determination of subchronic toxicity

The growth rate was reduced (fig. 2 and 3). The weights and macroscopic appearances of various organs (heart, spleen, liver, kidneys and thyroid glands) did not differ from those of the control animals. Histological examination of liver, kidneys, thyroids, adrenals, pituitary glands and bone marrow did not reveal any signs of toxicity. The weights of the adrenals increased in the males, and the weights of testes, ovaries and

[1]) *17-β-Oestradiolbenzoate*, Max Research Laboratories, Inc. New York = Oestradiolbenzoate.
Progesterone 1%, ACO, Stockholm = Progesterone.

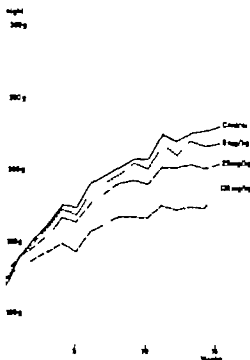


Fig. 2. Growth rate of young female rats after dosing by stomach tube 5 25 or 125 of mg/kg F6066 per day during 15 weeks. The growth rate is decreased and seems to be dependent on dose.

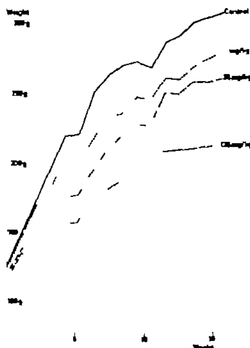


Fig. 3 Growth rate f young male rats fter dosing by stomach tube 5, 25 or 125 of mg/kg F6066 per day during 15 weeks. The reductio in nweight facrease is more pronounced than for the female rats.

Table 2

Pituitary inhibition test, vaginal smear test and test for uterotrophic effect of F6066, stilboestrol and oestradiol.

	Effective dose fo 45% decrease of (1) testes	(2) v prostate	Vaginal smear from female rats, ED50 (3)	ED uterotrophic effect mice (4)	Ratio (3)/(2)
F6066	0.3 mg	0.2 mg	0.78 mg	16 µg	3.9
Stilboestrol-dipropionate.	>1.6 µg	0.7 µg	0.48 µg	0.019 µg	0.7
Oestradiolbenzoate	0.4 µg	0.5 µg	0.24 µg	0.018 µg	0.5

5 rats per dose in the pituitary inhibition test, 20 ovariectomized rats per dose i the vaginal smear test and 10 immature mice per dose in the uterotrophic test were used.

prostates decreased, the latter to such a degree that they could hardly be found. Repeated haematological investigations (haemoglobin values, red and white blood cell count, differential count) did not reveal any departure from the normal blood picture.

Pituitary inhibition test vaginal smear test and test for uterotrophic effect

F6066 has an oestrogenic effect, but one only about $^1/_{1000}$ th those stilboestrol and oestradiol. The inhibitory effect of the substance on testes and ventral prostate is relatively stronger than that of conventional oestrogens (table 2)

In the test for uterotrophic effect, the activity-pattern is different for F6066 and the potent oestrogens, partly in that the maximal dryweights of uterus (obtained with 10 times the doses mentioned in table 2) differ with F6066 4 7 with stilboestrol 6.7 and with oestradiolbenzoate 5.8 mg and partly in that the uteri from animals treated with F6066 had a whiter and fatter appearance than those from the others.

Differences between F6066 and the potent oestrogens were seen even in the Allen-Doisy test, because, besides the ordinary cornified cells in an oestrus smear after F6066 dosing, more non-cornified epithelial cells were present than after dosing with stilboestrol and oestradiolbenzoate.

Absorption

F6066 has a uterotrophic effect by both the subcutaneous and the oral route. The effect after oral administration was greatest when the substance

Table 3

Absorption of F6066 after oral and subcutaneous administration to immature female mice.

	Administration	Body weight g	Dry uterus weight mg	s	1)
F6066 dissolved in 0.1 ml olive oil	sc	10.2	3 37	0.89	72
	po	9 7	2.67	0 61	57
F6066 dissolved in 0.1 ml corn oil	sc	10.5	3 22	0 69	69
	po	10.3	2.75	0.28	59
F6066 susp. in 0.1 ml gum arabic	sc	10 4	3.59	0.80	77
	po	10.2	1.69	0..5	36
Oestradiolbenzoate, 0.025 µg, dissolved in 0.1 ml olive oil	sc	10 8	4.66	0.60	100
Control 0.1 ml olive oil	sc	11 1	1 27	0.56	27
F6066 dissolved in olive oil	po	12.1	3 17	0.51	62
F6066 microcrystals susp. in gum arabic	po	12.1	2.57	0.38	48
F6066 macrocrystals susp. in gum arabic	po	12..	2.13	0.39	39

1) Percentage of the uterus dry weight from 0.025 µg oestradiolbenzoate.
s = standard deviation 10 animal per dose were used.

was dissolved in oil. When a suspension was given, the effect was most pronounced from microcrystals (table 3)

Growth and appetite

The growth rate corresponds to what was found in the earlier trial the dosage chosen here – 2 mg per day per animal subcutaneously in olive oil 5 times per week – corresponds to an oral dose of between 5 and 25 mg per kg in gum arabic daily (10 animals per sex and dose level), fig. 4

The appetite decreased to 60–80 / of the normal (fig. 5 and 6) The effect persisted 3 days after the injection. In a corresponding test on hypophysectomized, previously trained male rats (unpublished results from our laboratories) it proved difficult to obtain stable control values, but nothing in the tests indicates an anorexigenic effect here of F6066 or

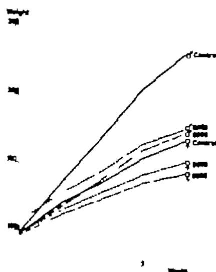


Fig. 4 Weight increase in young male and female rats after subcutaneous injections of a fixed dose (2 mg per day per animal) f F6066 F6060. The rate of increase is depressed by both substances.

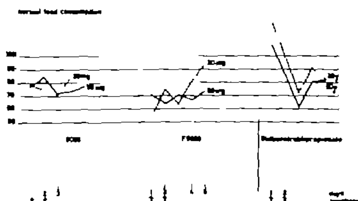


Fig. 5 Anorexigenic effect of F6066, F6060 and stilboestrol on adult trained female rats. Eating period 2 hours with 10 animals per dose. Dose expressed as per kg day

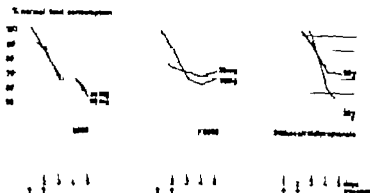


Fig. 6. Anorexigenic effect of F6066, F6060 and stilboestrol on adult trained male rats. Eating period 2 hours with 10 animals per dose. Dose expressed as mg per kg per day

stilboestrol, though dexamphetamine is still effective under the same conditions.

Reproduction

F6060 and *F6066* produce a reversible sterility in males and females (fig. 7 and 8) The sterility persists for a few weeks after discontinuing administration. One female continued to be sterile for more than 14 weeks after F6060 was with-drawn and one male was sterile for more than 11

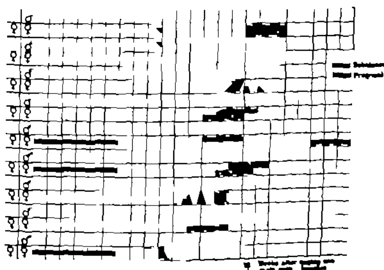


Fig. 7 Reproduction of rats after subcutaneous injections of 2 mg F6060 per animal 5 times a week for 6-9 weeks. The male or female rats were sterile for the injection period and for 1 15 weeks after but all except one female became pregnant.

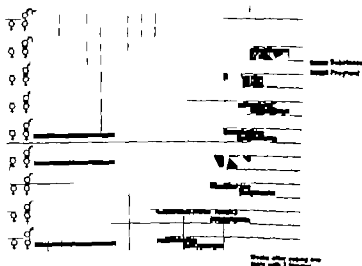


Fig. 8. Reproduction of rats after subcutaneous injection of 2 mg F6066 per animal 5 times a week for 6–9 weeks. The male or female rats were sterile for the injection period and for 3–9 weeks after but all except one male (or 2 females) from the first cage were fertile.

weeks after dosing with F6066, but this is within the limits of infertility in our rat-colony

Anabolic effect on castrated male rats

There was no sign of any anabolic effect of F6066 on castrated rats. Oestradiolbenzoate and stilboestrol have a weak anabolic effect on castrated animals. None of the substances changed the weight of the *musculus levator ani* in non-castrated rats (table 4).

Table 4

Anabolic effect of F6066, stilboestrol and oestradiol in castrated male rats.

	M. levator ani in mg				Body weight when killed, g
	wet	s	dry		
Olive oil	44	6	10.1	2.1	67.7
1 µg stilboestroldipropionate	69	10	17.6	4.4	73.6
1 µg oestradiolbenzoate	68	8	17.0	4.5	74.5
Olive oil	52	4	10.3	1.9	74.4
1.0 mg F6066	51	4	11.6	1.6	72.8
1.0 mg F6060	49	7	10.6	2.7	[illegible]

The test were performed in 2 different weeks on 5 rats per dose. s = standard

Table 5

Effect on lactation after administration of F6066 or oestradiol + progesterone to lactating rats.

Substance	Dose/day/animal	Weight of young at test start (g)	Weight on 5th day	Weight on 8th day	Number of dead young
Oestradiolbenzoate	0.1 mg l				
Progesterone	1.0 mg l	326	396	359	17
Olive oil		335	467	560	4
F6066	5.0 mg	259	395	481	6
Olive oil		284	358	417	6

The test were performed in 2 different weeks on 5 lactating rats in each group.

Lactation

There was no evidence of decreased lactation in rats from the doses of F6066 used (table 5)

Diuretic effect

F6066 has no diuretic effect during administration, but diuresis and Na excretion increased 3 days after administration of the second dose (table 6)

Table 6

Diuretic effect of F6066 in rats.

Urinary excretion	24 hours		48 hours		72 hours	
	Males	Females	Males	Females	Males	Females
Diuresis	1.3	1.3	1.4	1.5	1.4	0
Na	0.9	0.8	1.3	1.3	1.[illegible]	2.0
K	1.0	0.8	0.9	0.9	0.7	1.3
Cl	1.1	1.0	1.2	0.8	0.7	1.3
Creatinine	0.9	1.0	0.9	1.1	0.8	1.1

Relative values, the values of the corresponding control group being taken at 1.0. Each group consists of 4 animals.

Table 7

Antiinflammatory effect f F6066 and prednisolone after implantation of cotton pellets into adrenalectomized rats.

Mg/day	Body weight at slaughter	Pellets + granuloma, wet, mg per animal		Pellets + granuloma, dry mg per animal	
			s		
Olive oil	165	93	24	17.8	7.0
0.5 Prednisoloneacetate.	125	47	9	10.0	1.5
5.0 F6066	133	89	19	20.5	4 0
5.0 F6066 + 0.5 Prednisoloneacetate	123	50	13	10.7	3.0

Control values taken as unity s = standard deviation.

Anti-inflammatory effect

No anti-inflammatory effect or antagonism against prednisolone could be detected from the doses used (table 7)

Anti-gestational effects

If 0 5 mg/kg F6066 or more was injected simultaneously with 0.2 mg/kg progesterone no effect of the latter appeared in histological sections of the uterus. If 5 mg/kg F6066 were given only during the last two days before slaughter after progesterone injections, the typical secretory mucosa could be made partly to regress (fig. 9 and 10)



Fig. 9 Uterus from an immature rabbit injected on days 1 7 with 0.5 γ oestradiolbenzoate and days 8–14 with 0.. mg progesterone per animal. Fixation on day 15. A typical secretory endometrium is seen.

method of HERSBERGER, SHIPLEY & MEYER (1953) for demonstrating an anabolic effect. Even if this test may not be specific and has recently been subject to criticism (HAYES 1965), it shows that there is a *difference* between the action of F6066 and conventional oestrogens, in that the latter causes a growth of *musculus levator ani* in the castrated animals.

As shown in the lactation test, the LTH does not seem to be depressed by the dose of F6066 used.

In primates (FISHER & ZUCKERMANN 1937), in dogs (DANCE, LLOYD & PICKFORD 1959) and in rats (ZUCKERMANN PALMER & HANSSON 1950), the water retaining effect of natural oestrogens and stilboestrol has been demonstrated. The same thing can be shown with F6066 given in large doses (170 mg/kg) In the diuresis test on rats it is shown by the fact that the urinary excretion of sodium increases when the effect of the injected substance declines.

In an autoradiographic study on mice with ^{14}C-labelled F6066, HANNGREN, EINER JENSEN & ULLBERG (1965) have demonstrated a selective high accumulation in the corpora lutea and yolk-sac. This accumulation in corpora lutea, the antigestational properties in Claugberg's test and the antifertility properties when injected 5 times a week into rats suggest that F6066 and similar compounds may be useful as antifertility agents when given in the period after ovulation but before the placenta begins progestagen production.

Summary

1 F6066 is absorbed by both subcutaneous and oral routes in rats and mice.

2. The acute oral toxicity is low in mice.

3 No signs of toxicity were found in a 15 weeks test for chronic toxicity in rats, but some inhibition of growth and appetite was observed.

4 An uterotrophic effect in mice and a vaginotrophic effect in rats were found. These are recognized criteria of oestrogenicity but the doses required were large compared with those of normal oestrogens.

5 F6066 causes a decrease in the weight of testes and prostate. It has a more favourable ratio of pituitary inhibiting effect to oestrogenicity than the conventional oestrogens.

6. F6066 has no anabolic effect in castrated male rats.

7 F6066 has anti-gestational properties in rats and rabbits. A reversible sterility can be achieved in both male and female rats.

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The Effect of Prenylamine (Segontin®) on the Amine Levels of Brain, Heart and Adrenal Medulla in Rats

By

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Prenylamine, a derivative of amphetamine (prenylamine is N 3 propyl-(2')-1 1-diphenyl-propyl-(3)-amine) was first reported to the concentrations of noradrenaline (NA) and 5-hydroxytry... (5-HT) in the brain and heart of rats (SCHÖNE & LINDNER 1960).

Later the same authors (1962) showed that prenylamine also lowered the amine levels of the adrenal medullary granules *in vitro* Several workers have confirmed this and also shown that prenylamine blocked the uptake of amine by the adrenal medullary granules at small concentrations (CARLSSON HILLARP & WALDECK 1963 EULER, STJÄRNE & LISHAJKO 1964) These observations suggested that prenylamine might have reserpine-like actions.

The fact that the available data on the effect of prenylamine on monoamine metabolism are incomplete (for instance there were none on the effect on dopamine (DA) levels) and that prenylamine might prove a new pharmacological tool in the studies of central and peripheral mechanisms involving monoamines prompted the investigation.

While this manuscript was in preparation, two publications have appeared (JUORIO & VOGT 1965 MACKENNA 1965) reporting similar findings.

Methods

Male Sprague-Dawley rats weighing between 230 and 350 g were used. Prenylamine lactate, calculated as base, was dissolved in 5% glucose to give concentrations of 3 mg/ml or 5 mg/ml and administered by i p. injection.

The rats were beheaded, and the brains and hearts from two rats were pooled for each determination.

The hearts were cut open, blood was removed with filter paper and they were cut into small pieces with scissors. For each determinatio the tissues were ext acted with 15–18 ml ice-cold 0.4 N perchloric acid in a metal homogenizer (Ultra Turrax). The precipitate was spun down in a refrigerated centrifuge at 9000 × g for 10 minutes. After filtration of the supernatant, the residue was re-extracted with 10–12 ml ice-cold 0.4 N perchloric acid, and the two extracts were mixed.

When 5-HT was to be determined 0.1 ml 2 % ascorbic acid was added to every 10 ml of perchloric acid before extraction. This improved the recoveries of 5-HT (see below).

The extracts were divided into two parts, one for determining NA and DA and the other for 5-HT All determinations wer made fluorimetrically in the Aminco-Bowman spectrophotofluorimeter

NA and DA estimations

The extracts, which in most cases were frozen overnight at −20° were thawed in cold water 0.1 ml 2 % ascorbic acid and 0.2 ml 10 % EDTA (titracemin) being added to every 10 ml extract. They were then neutralized with 5 N potassium carbonate solution to pH 6.5 and applied to the column of the resin Dowex 50–x8 as described by BERTLER, CARLSSON & ROSENGREN (1958).

The elutio was performed with N-HCl, the first 2 ml being discarded. The next 8 ml contained the NA and the next 12 ml the DA.

NA was determined by the method of BERTLER, CARLSSON & ROSENGREN (1958) and the DA by that of CARLSSON & WALDECK (1958) as modified by CARLSSON & LINDQVIST (1962).

5-HT determinations

The extracts were neutralized with 5 N potassium carbonate to pH 6.5 and applied to the column of the resin, Amberlite XE-64 or Amberlite CG-50 (type 2) as described by BERTLER (1961).

After eluting the 5-HT with 3 ml N-HCl its determination was by the modified method of ANDÉN & MAGNUSSON (unpublished method).

The modification essentially involves treating the eluate as described below Ascorbic acid, 0.1 ml 1 %, was added to the standard, the reagent blank and 0.6 ml portions of the eluate (sample and internal standard) Ascorbic acid was not added to the 0.6 ml portion of the tissue blank. This was succeeded by addition of 0.1 ml 0.025 % $K_3Fe(CN)_6$ solution and 0.8 ml 6 N HCl to all solutions (standard, reagent blank, sample, internal standard and tissue blank).

They were then irradiated with U V light for 10 minutes, 0.1 ml 1 % ascorbic acid being added to the tissue blank immediately after irradiation. The reading was done as described by BERTLER (1961).

This addition of ascorbic acid to the perchloric acid bef re extraction (ANDÉN & MAGNUSSON) considerably prolonged the interval during which the determinatio could be made after extraction. It appears that ascorbic acid hinders the destruction of 5-HT in the presence f acid.

Determination of NA and A of the adrenal medulla

The adrenals from each rat were quickly removed, freed of connective tissues and fat and homogenized in 10 ml ice-cold 0.4 N perchloric acid in a glass homogenizer

The precipitate was spun down in a refrigerated centrifuge at 9000 × g for 10 minutes and filtered. The filterate was analysed for NA and A by differential reading by the method of BERTLER, CARLSSON & ROSENGREN (1958)

Recoveries

N corrections were made for the recoveries of known concentrations of the amines added to the extracts. These varied between 75% and 96% as indicated below:

NA – mean recovery = 91% (s.e.m. = 0.22 n = 10)
DA – – – = 84% (s.e.m. = 2.93 n = 9)
5-HT – – – = 85% (s.e.m. = 4.39 = 7)

Results

Effect of prenylamine on NA DA and 5-HT of the brain

The concentrations of NA, DA and 5-HT in the brains of no and the effect produced by intraperitoneal injection of preny shown in figs. 1 and 2. The DA concentration was reduced to about of its original value one hour after a single i p injection of preny 30 mg/kg (fig. 2) It remained at this level for about four hours and to about 70 / after 12 hours. At 72 hours the level had returned to

The NA levels followed a very similar pattern though the fall was as pronounced as that of DA This similarity in the pattern of the t curves suggested an identical action of prenylamine on the stores of the two amines, though NA stores appeared to be more resistant than those of DA.

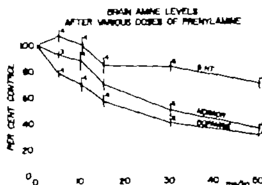


Fig. 1 Amine levels of rat brain 3 hours after i.p. injection of various doses of prenylamine. The amine levels of control rat brains were in µg/g wet tissue

for NA 0.441 ± 0.013 (16),
for DA 0.779 ± 0.024 (13), nd
for 5-HT 0.374 ± 0.013 (14).

The levels after various doses are expressed as percentages of the control levels. The vertical lines indicate the s.e.m. and the figures give the number of observations ( ).

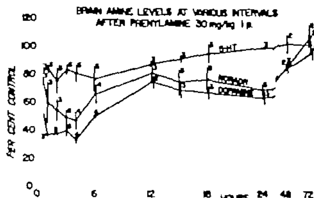


Fig. 2. Amine levels of rat brain at various intervals after i.p. injection of *prenylamine 30 mg/kg body weight. The indications are as in fig. 1*

There was only a slight apparent decrease in the 5-HT levels after prenylamine. Though this was observed in most of the experiments, only the decrease after a large dose (50 mg/kg) was statistically significant ($p = < 0.005$)

The effect of prenylamine on amine levels of the heart and the adrenal medulla

The values of the amine concentrations in the adrenal medulla and the heart of normal rats and after a single i.p. injection of prenylamine are given in figs. 3 and 4

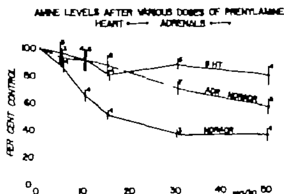


Fig. 3. Amine levels of the heart and adrenal medulla of rat 3 hours after i.p. injection of various doses of prenylamine. The control values were in µg/g wet tissue

NA of heart	0.807 ± 0.030	(19).
5-HT of heart	0.363 ± 0.038	(9).
NA of adrenal medulla	8.45 ± 0.77	(19).
A of adrenal medulla	22.01 ± 1.28	(19).

The indications are as in fig. 1

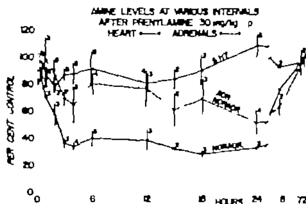


Fig. 4. Amine levels of the heart and adrenal medulla of rat at various intervals after i.p. injection of single dose of prenylamine 30 mg/kg body weight. The indications are as in fig. 1

The heart appeared to be more susceptible to prenylamine than the brain. *After 3 hrs. 10 mg/kg of prenylamine lowered the NA of the heart* to about 65 / and that of the brain only to 90 / The onset of action appeared to be the same in both tissues, but the recovery was much slower in the heart than in the brain Whereas the brain NA recovered to about 70 / of its normal level in 12 hrs. the heart NA level was below 40 / of its normal level and remained so at 24 hrs. (fig 2 and 4) As in the brain, the level had returned to normal at 72 hrs.

Of all the tissues examined, the adrenal medulla appeared to be the least susceptible to the effect of prenylamine. The onset of action was slower than in the brain and the heart, and also the level of the total amine fell only to about 60 / of the normal values, compared with 30 / and 45 / in the heart and the brain, respectively Only doses above 15 mg/kg were effective in lowering the catecholamine levels of the adrenal medulla, whereas 5 to 10 mg/kg reduced the amine levels in the brain and the heart. Prenylamine appeared to lower the A more than the NA concentration, and this might account for the slower recovery of A levels. It must be mentioned that there was a fairly wide variation in the relative amounts of NA and A of the adrenal medulla, and this may have masked the effect of prenylamine on the NA and A levels.

Discussion

The findings that in the rat prenylamine lowers the NA and DA levels of the brain, NA of the heart and NA and A of the adrenal medulla are consistent with those of SCHÖNE & LINDNER (1960) (NA of brain and

heart) JUORIO & VOGT (1965) (NA and DA of rat's brain) and MACKENNA (1965) (rabbit's heart).

Only the largest dose used (50 mg/kg) caused a statistically significant fall in the 5-HT levels after prenylamine. *Similar findings were reported* by SCHÖNE & LINDNER (1960) (cf JUORIO & VOGT who did not find a significant fall in the 5-HT levels after an exceptionally large dose, 100 mg/kg).

The slight apparent fall of 5-HT levels in the heart after prenylamine was not statistically significant. In all probability the 5-HT of the rat heart is derived from mast cells. It is known that mast cells are resistant to the amine-depleting action of reserpine since prenylamine has actions similar to those of reserpine, the ineffectiveness of prenylamine in this respect is not surprising.

There is another quantitative difference between our findings and those of JUORIO & VOGT. Whereas in their experiments the NA and DA levels of the brain were reduced to 75% and 40% of their respective normal values, our findings were that the levels of NA and DA were reduced to 45% and 35% respectively even though a lower dose was used. This discrepancy may possibly be accounted for by the different routes of administration of prenylamine in the two experiments. Throughout the present experiments, prenylamine *lactate* was dissolved in 5% glucose to give a concentration of 3 mg/ml or 5 mg/ml, and all injections were by i.p. route.

JUORIO & VOGT used prenylamine *gluconate* solution in their experiments and gave 50 mg/kg by the s.c. route, the solution being diluted with glucose (VOGT, personal communication). To check this, prenylamine gluconate solution was diluted with 5% glucose to give a concentration of 10 mg/ml, and 30 mg/kg were administered to rats by both i.p. and s.c. routes; the amine levels were then determined after 4 hrs. The i.p. route reduced the NA and DA levels by 25% and 15% respectively more than the s.c. route (2 experiments). The different routes of administration may thus account for part of the difference. An additional factor may be that JUORIO & VOGT analysed the hypothalamus only whereas in the present investigation the whole brain was used. It has been shown that the NA of the hypothalamus is more resistant to reserpine than that of the cortex (ANDÉN, personal communication).

The effect of prenylamine on the amine levels is relatively transient. After 6 hrs. both the DA and NA levels of the brain have begun to recover and after 72 hrs. are fully recovered. Similar findings were reported by JUORIO & VOGT though the recoveries in our experiments were more rapid. This is perhaps related to the different routes of administration.

Evidence is accumulating in support of the view that prenylamine has

similar (though milder) actions to reserpine. Actions of prenylamine on adrenal medullary granules (CARLSSON, HILLARP & WALDECK 1963 EULER, STJÄRNE & LISHAJKO 1964 PHILIPPU PALM & SCHUMANN 1965 LUNDBORG, 1966) are similar to those of reserpine The initial stage of block induced by reserpine is competitive and may thus be prevented by agents with the same point of attack, such as tetrabenazine (QUINN, SHORE & BRODIE 1959). *Prenylamine has also been shown partly to pre*vent the action of reserpine (CARLSSON 1965)

Nevertheless, there are differences, which may be quantitative rather than qualitative, between the actions of the two drugs. For instance, the only visible sign of drug effect after moderate doses of prenylamine in rats is muscular weakness, in contrast with the well known effects of As it is to reserpine, the heart is more sensitive to the effect of preny! than the brain, but, unlike the response to reserpine, the brain NA to recover earlier than that of the heart (cf CARLSSON ROSENG BERTLER & NILSSON 1957).

The milder effect of prenylamine could be due to a lower affinity f the sites of uptake in the storage granules. A more rapid elimination o prenylamine may also contribute. However tetrabenazine, which has transient effect does not have such mild one. Prenylamine marks the fir‑ of an entirely new type of amine-depleting agents and may form the basis for the synthesis of more potent substances in view of its relatively simple structure.

Efforts are continuing in this laboratory to elucidate the mechanism of action of prenylamine.

Summary

A single dose of prenylamine (30 mg/kg) given intraperitoneally lowered the brain DA and NA to less than 40% the NA of the heart to less than 30% and NA and A of the adrenal medulla to about 65% of the normal values in rats.

The apparent slight fall in the levels of 5-HT in the brain and the heart, though seen in almost all the experiments, was not found statistically significant. Although prenylamine has a different chemical structure from reserpine, there are many similarities in their actions.

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